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ADJUVANT EFFECTS ON IMMUNE RESPONSES TO BIOLOGIC AGENTS.(U)

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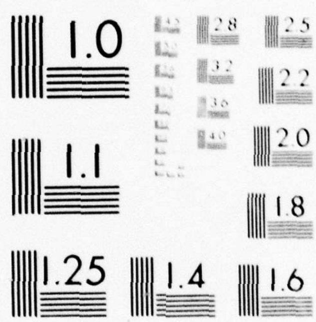

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SUMMARY

This report presents the progress on research supported by the Contract DAMD 17-74-C-4095 during the fiscal year 1978. Morphologic, immunologic and radiokinetic studies have been carried out to provide new insight into the bioregulation of lymphocyte traffic in normal animals and how this may be modified by adjuvants to potentiate immune responses to vaccines. The membrane determinants of lymphocyte "homing" are analyzed and evidence is presented for the cytoskeletal control of these cellular interactions. Data are presented supporting the concept that "scrubbing" of lymphocyte surfaces by high endothelial cells may be an important but previously unrecognized phenomenon for the successful initiation of <u>in vivo</u> immune responses. The possible role of chemotaxis in regulating the entry and redistribution of lymphocyte subpopulations into different anatomical regions of peripheral lymph nodes is analyzed. Studies of the mechanisms by which complete Freund's adjuvant enhances immune responses to VEE have been combined with surgical thymectomy to demonstrate that the ablation of T-cell suppressor effects can potentiate immune responses initiated by vaccine in adjuvant.

PUBLICATIONS

Johns Hopkins Med. Journal 141:135, 1977
Johns Hopkins Med. Journal (Submitted for publication)
Fed. Proc. 37:591A, 1978
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Cell. Immunol. (Submitted for publication)
Cell and Tissue Kinetics (Submitted for publication)

FOREWARD

This annual progress report is a summary of the research activities carried out by Norman D. Anderson, M.D. (Principal Investigator), Robert G. Hoffman, B.S. and Stephen C. Ponchuk, B.S. (Associate Investigators) at The Johns Hopkins University School of Medicine, Baltimore, Maryland. This work was supported by Contract DAMD 17-74-C-4095 and conducted under Project 3A762760A834-02-921, "Prevention and Treatment of Biologic Agent Casualties".

In conducting this research, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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INTRODUCTION

a) Research Objectives. The goals of this research are to define the biological mechanisms regulating immune responses in vivo and to utilize this information in developing new adjuvants for potentiating immune responses to marginal vaccines.

b) Background Information. There is convincing evidence that successful initiation of immune responses to most antigens depends upon a complex sequence of: antigen presentation; collaboration between T-lymphocytes, B-lymphocytes and macrophages; cellular proliferation, differentiation and mobilization which are modulated by suppressor cells, specific antibody and local microenvironmental changes induced by the release of lymphokines and macrophage products (1, 2, 3, 9). Since the effective use of adjuvants depends upon the appropriate manipulation of one or more of these events in vivo (3), precise definition of the mechanisms regulating unmodified immune responses remains as a prerequisite for understanding adjuvant-induced immunopotentiation.

Previous studies have established that organized lymphatic tissue, with its complex cellular populations, reticular meshwork, lymphatic and vascular connections provides an efficient locus for facilitating in vivo immune responses. There is ample evidence indicating that antigens entering through lymphatics are effectively bound and concentrated within regional lymph nodes which function as stationary filters set astride the lymphatic network. Since there are only 10^3 - 10^5 lymphocytes capable of reacting with a given antigen in unprimed animals, random movement of these cells through the body would be quite unlikely to yield the chance encounter necessary for immune recognition. This is clearly not the case in higher animals as Gowans (4) and others have established that immunocompetent T&B lymphocytes exhibit a preferential homing instinct for lymphoid organs and continually percolate through these antigen-binding filters as they recirculate between blood and lymph. This selective lymphocyte traffic provides a highly efficient means of immunosurveillance and can be regarded as one of the critical steps in initiating immune reactions in vivo. In attempts to resolve some of the mechanisms which regulate lymphocyte recirculation our previous progress reports have: (1) provided the first detailed descriptions of the lymph node micro-vasculature and the local hemodynamic control mechanisms which regulate the initial distribution of blood-borne lymphocytes within lymph nodes; (2) defined the route followed by lymphocytes migrating across HEV walls; (3) demonstrated that these intercellular spaces through which lymphocytes emigrate function as anatomical communications between lymph and blood permitting macromolecular tracers to flow from the node across the HEV wall into the venular lumens; (4) provided morphologic data indicating that lymphocytes actively migrate through these spaces showing directional movement from the venular lumen into the nodal parenchyma; (5) documented that selective attachment of lymphocytes upon HEV surfaces is mediated by specialized microvillus projections which interdigitate with pits on the endothelial surface; (6) demonstrated that heparin and other surface-active agents can prevent or block this selective surface recognition.

These findings have now been complemented by studies demonstrating that surface ligands can alter the lymphocyte surface (Project 1) and block lymphocyte traffic in vivo (Project 2). Further, we have found that these surface recognition mechanisms are subjected to cytoskeletal

CONTROL THROUGH TRANSMEMBRANE RESTRAINTS. (PROJECT 4). OUR RECENT STUDIES ALSO SUGGEST THAT HIGH ENDOTHELIAL CELLS IN LYMPH NODES HAVE SPECIALIZED PHAGOCYTIC CAPABILITIES NOT SEEN IN OTHER VESSELS WHICH SERVE TO "SCRUB" SURFACE LIGANDS AND ADSORBED PROTEINS FROM THE SURFACES OF EMIGRATING LYMPHOCYTES (PROJECT 3). DATA DESCRIBED IN PROJECT 5 PROVIDES AN IN VITRO MODEL WHICH APPEARS TO BE QUITE USEFUL IN STUDYING THE ROLE OF CHEMOTAXIS IN REGULATING LYMPHOCYTE TRAFFIC IN PERIPHERAL NODES. OUR PREVIOUS OBSERVATIONS THAT CHEMICALLY DISPARATE ADJUVANTS SHARE THE COMMON BIOLOGIC PROPERTY OF AUGMENTING CELLULAR TRAFFIC INTO REGIONAL NODES, HAVE NOW BEEN EXPANDED TO INCLUDE DETAILED OBSERVATIONS ON THE MECHANISMS OF IMMUNOPOTENTIATION BY COMPLETE FREUND'S ADJUVANT AND HOW THIS EFFECT CAN BE ENHANCED BY THYMECTOMY (PROJECT 6). WHEN ALL OF THESE RESULTS ARE COMBINED, THEY CERTAINLY SUGGEST THAT THERE ARE SEVERAL BIOLOGICAL MECHANISMS WHICH CAN BE MANIPULATED BY EXISTING METHODOLOGIES WHICH COULD BE OF REAL INTEREST IN ACHIEVING IMMUNOPOTENTIATION TO MARGINAL VACCINES.

PROJECT I: THE LYMPHOCYTE GLYCOCALYX AND ITS MODIFICATION BY SURFACE LIGANDS.

THE PRECISE MECHANISMS RESPONSIBLE FOR THE SELECTIVE ATTACHMENT OF RECIRCULATING LYMPHOCYTES TO HEV SURFACES ARE STILL CONTROVERSIAL. SINCE GESNER AND WOODRUFF (1969) FOUND THAT TREATMENT WITH DILUTE TRYPSIN COULD PREVENT LYMPHOCYTES FROM RECIRCULATING THROUGH PERIPHERAL NODES, IT HAS BEEN GENERALLY ASSUMED THAT SURFACE GLYCOPROTEINS MEDIATE THE SELECTIVE INTERACTIONS WITH HEV. WHILE THIS PREMISE MAY BE CORRECT, OTHER INVESTIGATORS (SINGER, 1975) HAVE STATED THAT SUCH FINDINGS CAN HARDLY BE CONSIDERED AS CONVINCING EVIDENCE FOR GLYCOPROTEINS SERVING AS SURFACE RECOGNITION MECHANISMS AS THERE IS AMPLE PROOF THAT PROTEASE TREATMENT CAUSES MANY MODIFICATIONS IN THE LYMPHOCYTE SURFACE SUCH AS CHANGES IN ZETA POTENTIAL, ALTERED CHARGE DISTRIBUTION AND RELEASE OF SURFACE RECEPTORS FROM PERIPHERAL TRANSMEMBRANE RESTRAINTS. THESE CRITICISMS HAVE BEEN ANSWERED, IN PART, BY OBSERVATIONS DESCRIBED IN OUR PREVIOUS PROGRESS REPORTS WHICH DEMONSTRATED THAT: (1) DILUTE TRYPSIN COULD ALSO DISLODGE ADHERENT LYMPHOCYTES FROM HEV WHICH SUGGESTED THAT THE SAME PROTEASE SENSITIVE SURFACE CONSTITUENTS WERE RESPONSIBLE FOR RECOGNITION AND ATTACHMENT, AND (2) OUR ULTRASTRUCTURAL OBSERVATIONS WHICH SHOWED THAT LYMPHOCYTES ATTACHED TO HEV THROUGH VILLUS PROJECTIONS WHICH INTERDIGITATED WITH FOCAL SURFACE DEPRESSIONS ON THE ENDOTHELIAL CELLS AND WHEN SURFACE COAT OLIGOSACCHARIDES WERE PRESERVED BY SPECIAL FIXATION TECHNIQUES, A FIBRILLAR ARRAY OF ELECTRON DENSE MATERIAL WAS SEEN BRIDGING THE INTRAMEMBRANOUS GAPS WITHIN THESE CONTACT POINTS. AS THESE MORPHOLOGIC FINDINGS PROVIDE THE BEST AVAILABLE EVIDENCE SUGGESTING THAT LYMPHOCYTE ATTACHMENT TO HEV SURFACES MAY BE DEPENDENT UPON SURFACE COAT INTERACTIONS, WE HAVE INITIATED STUDIES TO BETTER CHARACTERIZE THE OUTER STRUCTURE OF LYMPHOCYTE MEMBRANES.

OTHER INVESTIGATORS HAVE EMPLOYED SELECTIVE STAINING TECHNIQUES TO DEFINE A GLYCOPROTEIN-RICH SURFACE COAT EXTERNAL TO THE PLASMA MEMBRANE IN A WIDE VARIETY OF CELL TYPES WHICH HAS BEEN POSTULATED TO PLAY IMPORTANT ROLES IN CELLULAR RECOGNITION, DIFFERENTIATION, CONTACT INHIBITION, ETC. IN VIEW OF RECENT BIOCHEMICAL STUDIES OF MEMBRANE COMPOSITION, THESE STAINING PROCEDURES PROBABLY DEFINE COMPLEX POLYSACCHARIDES, PROTEOGLYCANS AND GLYCOPROTEINS WHICH ARE ANCHORED TO THE PLASMA MEMBRANE THROUGH HYDROPHOBIC "TAILS" IMBEDDED IN THE LIPID BILAYER AS WELL AS PERIPHERAL MEMBRANE CARBOHYDRATES WHICH ARE LINKED TO INTEGRAL COMPONENTS BY HYDROGEN BONDING, SALT BRIDGES OR OTHER FORCES. THE INTERACTIONS OF CATIONIC DYES OR SPECIFIC LIGANDS WITH THESE SURFACE CARBOHYDRATES PROVIDES A UNIQUE OPPORTUNITY TO STUDY OUTER MEMBRANE STRUCTURE AND ITS PUTATIVE LINKAGE TO

THE LYMPHOCYTE CYTOSKELETON BY ULTRASTRUCTURAL TECHNIQUES.

WE ARE CURRENTLY CARRYING OUT SUCH STUDIES IN COLLABORATION WITH MAJOR ARTHUR ANDERSON AT USAMRIID USING 4 DIFFERENT AGENTS WHICH REACT WITH LYMPHOCYTE MEMBRANES. THE RESULTS OF THESE EXPERIMENTS ARE DETAILED BELOW.

A) RUTHENIUM RED. THORACIC DUCT LYMPHOCYTES WERE COLLECTED FROM BOLLMAN-TYPE FISTULAS IN NORMAL LEWIS RATS. AFTER WASHING ONCE, THESE CELLS WERE DIVIDED INTO 2 SAMPLES. ONE ALIQUOT WAS ADDED DIRECTLY TO PHOSPHATE-BUFFERED 3% GLUTARALDEHYDE CONTAINING 0.05% RUTHENIUM RED AND PROCESSED FOR ELECTRON MICROSCOPY. THE REMAINING CELLS WERE INCUBATED IN CULTURE MEDIUM CONTAINING 0.05% RUTHENIUM RED FOR 10 MINUTES AT 37°C, WASHED, AND CULTURED IN M-199 SUPPLEMENTED WITH 10% RAT SERUM. AT SEQUENTIAL TIME INTERVALS RANGING FROM 2 MINUTES TO 24 HOURS, REPRESENTATIVE CULTURES WERE HARVESTED, WASHED AND FIXED IN PHOSPHATE-BUFFERED GLUTARALDEHYDE. THE RESULTS OF THESE STUDIES HAVE SHOWN THAT THE ADDITION OF RUTHENIUM RED DURING FIXATION RESULTED IN THE LYMPHOCYTES BEING COVERED BY A THIN LAYER OF ELECTRON DENSE MATERIAL MEASURING 90 - 270A° IN THICKNESS WHICH SURROUNDED THE ENTIRE PERIPHERY OF MOST CELLS. IN A FEW LYMPHOCYTES THIS LAYER WAS INTERRUPTED IN SOME POINTS BY GAPS OF UNLABELED MEMBRANE WHICH CORRESPONDED TO PROTRUSIONS OR MICROVILLI. THIS RATHER UNIFORM STAINING OF THE LYMPHOCYTE GLYCOCALYX WITH RUTHENIUM RED CHANGED DRAMATICALLY IF THE CELLS WERE CULTURED PRIOR TO FIXATION. CELLS FROM THE 2-10 MINUTE CULTURES SHOWED THAT THE RUTHENIUM RED-STAINED GLYCOCALYX WAS NOW BROKEN UP INTO A FUZZY, UNEVEN LAYER INTERSPERSED BETWEEN LARGE SEGMENTS OF UNSTAINED MEMBRANE. BY 30 MINUTES, VIRTUALLY ALL LYMPHOCYTES DISPLAYED RUTHENIUM-RED GLYOCALYCEAL COMPLEXES CONCENTRATED AS A BROAD CAP OVER THE UROPOD. WHILE OUR STUDIES ON THE SUBSEQUENT FATE AND RATE OF CLEARING OF THESE MEMBRANE CAPS ARE STILL IN PROGRESS, PRELIMINARY OBSERVATIONS SUGGEST THAT THE RUTHENIUM-RED TRACER IS SLOWLY CLEARED FROM THE LYMPHOCYTE CAP BY ENDOCYTOSIS AND SHEDDING OVER THE NEXT 4-6 HOURS. EQUALLY INTERESTING WERE OBSERVATIONS THAT THIS SEQUENTIAL PROGRESSION FROM DIFFUSE COATING TO PATCH AND FINALLY CAP FORMATION WERE PARALLELED BY CYTOSKELETAL CHANGES. IN LYMPHOCYTES WHERE THE GLYCOCALYX WAS STAINED DURING FIXATION, A NETWORK OF THIN MICROFILAMENTS WAS SEEN EVENLY DISTRIBUTED IN THE PERIPHERAL CYTOPLASM JUST BENEATH THE PLASMALEMMA. WHEN THE CELLS WERE FIXED AT 10 MINUTES AFTER EXPOSURE TO RUTHENIUM RED, THICKER LAYERS OF THE SUBMEMBRANOUS MICROFILAMENTS WERE SEEN BENEATH THE SURFACE PATCHES WHILE THE DELICATE NETWORK OF SUBPLASMALEMMAL MICROFILAMENTS APPEARED UNALTERED BENEATH THE INTERVENING SEGMENTS OF MEMBRANE DEVOID OF SURFACE STAINING. WHEN CAP FORMATION APPEARED AFTER 30 MINUTES, WIDE BUNDLES OF MICROFILAMENTS WERE FOUND CONCENTRATED WITHIN THE UROPOD IN A DEEPER LOCATION EXTENDING FROM THE GOLGI REGION TOWARDS THE CAPPED MEMBRANE. THIS COMPLEMENTARY REDISTRIBUTION OF CYTOPLASMIC MICROFILAMENTS AS RUTHENIUM RED PATCHES AND CAPS ON THE LYMPHOCYTE SURFACE CERTAINLY SUGGESTS THAT AT LEAST SOME PORTIONS OF THE LYMPHOCYTE GLYCOCALYX ARE LINKED THROUGH THE MEMBRANE TO THE CYTOSKELETON. THIS CONCEPT HAS BEEN DIRECTLY APPLIED TO IN VIVO CELL TRAFFIC IN STUDIES DESCRIBED IN LATER SECTIONS OF THIS REPORT WHERE PHARMACOLOGIC AGENTS WHICH DISRUPT THE CYTOSKELETON ARE SHOWN TO BLOCK LYMPHOCYTE ENTRY INTO PERIPHERAL NODES.

B) ALCIAN BLUE. THE SAME GENERAL EXPERIMENTAL DESIGN DESCRIBED ABOVE HAS BEEN FOLLOWED IN USING ALCIAN BLUE DYE TO STAIN THE LYMPHOCYTE GLYCOCALYX. WHILE COMPARABLE CONCENTRATIONS OF THIS DYE

(0.5 - 0.05%) caused overt cytotoxicity readily demonstrated by trypan blue exclusion, the use of .002 - .0002% alcian blue has consistently yielded diffuse staining of the lymphocyte surface coat which progressed to patch and cap formation within 30 minutes with identical patterns of reorganization of the cytoplasmic microfilaments. These observations clearly establish that these changes in lymphocyte structure can be generalized to a variety of different cations which react with surface carbohydrates.

c) Ferritin-Conjugated Concanavalin A. Other investigators (Yahara and Edelman, 1974, Karnovsky and Unanue, 1974) have reported that the plant lectin - concanavalin A selectively binds to mannoside residues in the carbohydrate side chains of glycoproteins, and at high concentrations this interaction can inhibit the patching and capping of other surface antigens. While there still is some debate over whether this inhibition of capping is due to anchoring of the surface receptors through a membrane modulating system or simply the direct result of crosslinking of the various membrane components by Con A, this lectin certainly provides a means for covering membrane glycoproteins which might prevent surface recognition between lymphocytes and high endothelial cells.

Because of our interest in such potential "blindfolding" effects, we have begun a series of studies on the ultrastructural changes induced in rat thoracic duct lymphocytes by concanavalin A. While high concentrations of this lectin (< 20 µg/ml) can coat the outer lymphocyte membrane to produce a thin layer of increased radiodensity demonstrable by high resolution electron microscopy, we have purposely avoided this approach as such dosages also produce rapid cell death (de Petris, 1975). Instead, we have labeled highly purified Con A with ferritin using techniques described by Edelman (1973). Then rat thoracic duct lymphocytes were incubated in vitro with non-cytotoxic concentrations of this reagent (1-10 µg/ml) for 10 minutes at 37°C. The cells were then washed; returned to culture media for intervals ranging from 3-120 minutes, and then fixed in glutaraldehyde and processed for electron microscopy. Several interesting features have evolved from these studies. In each sample examined, this procedure has yielded surface labeling of 90-98% of all the lymphocytes by scattered ferritin particles distributed as single granules over the membrane or coalesced patches of multiple ferritin particles. Diffuse, homogeneous labeling comparable to that produced by the metal dyes has never been seen. Further, when these cells are examined after progressive time intervals of culture, there is a definite tendency for the ferritin tracer to aggregate into discrete focal patches but frank capping has been observed in only 4-6% of the cells. While this makes it difficult to determine whether Con A induces a reorganization of the lymphocyte cytoskeleton; these patches appear to persist for hours with only minimal signs of their being cleared by shedding or endocytosis. Such findings suggest that this lectin may be an ideal agent for determining the effects of membrane "blindfolding" upon lymphycyte traffic in vivo as shown in Project 2.

d) Ferritin and Peroxidase Conjugated Anti-Immunoglobulin. There is an extensive literature demonstrating that tracer-conjugated anti-immunoglobulins bind selective to lymphocyte surface IgG to produce patching and capping. To permit direct comparisons with observations made with the cationic dyes and Con A, we have also undertaken

A BRIEF EVALUATION OF THE ULTRASTRUCTURAL CHANGES INDUCED IN LYMPHOCYTES BY ANTI-IMMUNOGLOBULINS.

Rabbit-anti-rat IgG conjugated with either horseradish peroxidase or ferritin was purchased from Cappell Laboratories (Downingtown, Pa.). After absorbing this material twice with an equal volume of freshly isolated Lewis rat thymocytes to remove possible species-specific antibodies, the conjugated globulin was diluted to a concentration of 50 mg. protein per ml. This adsorbed antibody was then reacted with rat thoracic duct lymphocytes (which had been washed x3 to remove extraneous proteins) for $\frac{1}{2}$ hour at $5^{o}C$. The cells were then centrifuged, washed, and transferred to culture medium. After incubation at $37^{o}C$ for varying time intervals, the cells were harvested and processed for EM studies using methods described previously. Electron micrographs of these lymphocytes showed the typical sequence of scattered diffuse labeling of the outer membrane surface by electron dense tracers in 1/3 of the thoracic duct cells which rapidly progressed during incubation at 37^{o} to patch and cap formation at $\frac{1}{2}$ hour. At later intervals (2-4 hours) the lymphocyte surfaces were completely free of tracer, but ferritin granules could be demonstrated within endocytic vacuoles and extracellular debris. Of equal interest in this study, were observations that nuclear pores tended to lie directly beneath microvillus projections in cells which had not yet developed large patches or caps. Most of these microvilli contained a central core of aligned microfilaments passing along their longitudinal axis, and in some cells these filaments or the surrounding zone of ribosomal exclusion could be traced through the cytoplasm to sites at or near the underlying nuclear pore. As patching progressed to capping, the microfilaments appeared to be reorganized into broad bundles concentrated in the cytoplasm beneath capped membrane of the uropod. In these cells the nucleus appeared to be rotated or reoriented so that the nuclear pores were all concentrated on the pole of the nucleus facing the uropod while the paranuclear cystern dominated the opposite nuclear pole. These observations are particularly intriguing for they certainly suggest that alterations in the outer lymphocyte membrane are transmitted into cytoskeletal reorganization with concomitant changes in nuclear morphology. Since this ligand is known to be mitogenic, these findings may provide the cytostructural basis on how membrane signals are transmitted to the nucleus. Because of these implications further study of this phenomenon is in progress.

Project 2. The Blockade of Lymphocyte Recirculation by Surface Ligands. (The "Blindfolding" Hypothesis).

In the mid 1960's, Medawar postulated that coating or covering of lymphocyte surfaces by non-toxic agents might disrupt lymphocyte recirculation by "blindfolding" the membrane segments required for surface recognition. Subsequent attempts to document the val.dity of this concept for explaining the altered lymphocyte traffic kinetics produced by anti-immunoglobulins, lectins and mitogens have been inconclusive. Since the observations outlined in Project I have defined several different agents which cause diffuse and prolonged coating of lymphocyte surfaces, we have combined this metholology with quantitative measurement of radiolabeled lymphocyte traffic kinetics to determine whether "blindfolding" by surface ligands can block lymphocyte recirculation *in vivo*.

a) <u>Studies with Ruthenium Red</u>. The <u>in vitro</u> effects of varying concentrations of ruthenium red dye upon rat lymphocytes are summarized in Table 1. These results show that 5×10^6 rat spleen cells cultured for 72 hours with ruthenium red at dosages of (0.01 - 1.0) mg/ml failed to incorporate ^{3}H-thymidine at levels indicative of a mitogenic response. High dye concentrations (1.0 mg/ml) caused immediate agglutination and direct toxic effects on thoracic duct lymphocyte suspensions. However, these changes were not seen when the dosage of ruthenium red was reduced to 0.1 - 0.5 mg/ml. Despite the production of surface patches and caps demonstrable by TEM, rat lymphocytes treated with low dye concentrations displayed normal patterns of random and directional movement when their migration in agarose plates was assayed against endotoxin activated serum.

Direct assessment of the possible cell traffic changes induced by coating lymphocyte surface with ruthenium red was then made using techniques described in our previous progress reports. After labeling rat thoracic duct lymphocytes by <u>in vitro</u> incubation with ^{3}H-uridine, these cells were washed and exposed to varying concentrations of ruthenium red for 10 minutes at 37°C. Then the cells were washed again, resuspended in culture medium and injected intravenously into normal Lewis rats at a dose of 10^6 per gram body weight. The recipients were killed at sequential intervals post-infusion and their viscera excised, weighed and processed for whole organ counting. The results of these studies are summarized in Table 2 which shows that cytotoxic dye concentrations (1 mg/ml) caused a marked suppression in the accumulation of radiolabeled lymphocytes in lymphoid organs which was paralleled by increased hepatic uptake of radioactivity. When the ^{3}H-uridine labeled cells were exposed to ruthenium red at doses of 0.05 mg/ml before infusion, their entry into peripheral nodes was reduced by 30-40% at each time interval studied. While this could reflect "blindfolding", this reduction in radioactivity within lymphoid tissues can be accounted for by the enhanced hepatic uptake of ^{3}H-uridine seen in the same animals. Since dead or severely damaged lymphocytes tend to sequester in the hepatic RE system after transfusion, the above findings can best be explained by ruthenium red induced cytotoxicity which was not detected by dye exclusion methods. This concept is supported by our observations that lymphocytes treated with ruthenium red at 0.01 mg/ml showed identical patterns of patching and capping of their surface components, but when these cells were infused <u>in vivo</u> they displayed completely normal traffic kinetics in lymphoid organs. When combined with results presented in Project 1, these findings indicate that ruthenium red can combine with diffuse surface coat constituents without affecting the ability of lymphocytes to recirculate <u>in vivo</u>. This negates simplistic theories of cellular "blindfolding", but it does not preclude a role for surface coat in the attachment sequence as most of the lymphocyte membranes are cleared of dye as the complexes aggregate and cap over the uropod. Ultrastructural studies are in progress to clarify this issue by determining whether ruthenium red treated-lymphocytes do, in fact, attach to HEV surfaces and emigrate across the venular wall in a normal manner.

b) <u>Studies with Alcian Blue Dye</u>. The effects of graded doses of alcian blue dye upon rat lymphocytes in various <u>in vitro</u> assays are shown in Table 1. This surface ligand failed to produce direct mitogenic effects upon spleen cell cultures. At concentrations of

200 ug/ml Alcian blue caused lymphocyte aggregation and direct cytotoxicity within 10 minutes, but both of these effects disappeared at lower dosages which induced surface patching and capping without causing detectable changes in cellular motility. In vivo traffic studies (Table 4) employing ^{3}H-uridine labeled lymphocytes demonstrated that brief incubation with Alcian blue at 200 ug/ml markedly suppressed the accumulation of these cells within lymphoid organs. As expected from the viability studies, this change was clearly due to direct lymphocyte cytotoxicity reflected by the striking increase in the hepatic uptake of radioactivity in these rats (16.9% as opposed to the 5%-10% range seen after infusing normal cells). However, similar treatment of radiolabeled lymphocytes with Alcian blue at 20 μg/ml caused an almost equivalent suppression of cell traffic into lymphatic tissues. Since this was not paralleled by increased hepatic radioactivity or overt toxicity demonstrable by dye exclusion techniques, this pattern of altered recirculation can probably be attributed to "blindfolding". As lymphocytes exposed to lower dye concentrations (0.2 - 2.0 ug/ml) displayed normal traffic patterns in vivo, the ability of Alcian blue to block recirculation appears to be dependent upon a critical dosage range. The precise membrane-cytoskeletal changes induced by 20 ug/ml of Alcian blue are still being studied by electron microscopy (Project 1). Our preliminary data suggests that the dye-glycocalyx complexes are cleared from the lymphocyte surface within 4-6 hours. If this is confirmed, the prolonged suppression of lymphocyte recirculation seen in vivo may reflect the time required for the treated lymphocytes to regenerate new membrane recognition sites.

c) Studies with Concanavalin A. In vitro observations on rat lymphocytes treated with tetravalent Concanavalin A are presented in Table 5. The results indicate that standard doses of this lectin produce mitogenic responses in rat lymphocytes without altering cell viability or inducing agglutination. When the Con A dosage is increased to 2.5-10 μg/ml, this produces overt cytotoxicity with loss of the mitogenic effects. All of the Con A dosages tested in this study caused patch formation demonstrable by electron microscopy (Project 1) but this rarely progressed to cap formation when thoracic duct lymphocytes were used as the cell source. Of equal interest, are the observations that both conventional and high Con A dosages appeared to suppress lymphocyte motility as measured by migration under agarose. Results presented in Table 6 compare the lymphocyte traffic kinetics seen after treating radiolabeled lymphocytes with varying concentrations of Con A prior to their infusion into normal recipients. The complete failure of lymphocytes exposed to 5-10 ug/ml Con A to accumulate in peripheral lymph nodes may be due to ligand-induced membrane changes since there was no evidence for increased hepatic uptake of the label usually seen with death of the infused cells. Further, lymphocytes pre-incubated with 2.5 μg/ml Con A exhibited a 50% reduction in their ability to enter lymphatic tissues in the absence of any signs of cytotoxicity. Since comparable cells treated with 1 μg/ml Con A displayed entirely normal traffic kinetics in vivo, this blockade of lymphocyte recirculation appeared to be dose related. When these observations are combined with evidence from other laboratories indicating that there are a variety of surface receptors with different affinities for Con A, it seems entirely plausible that increasing concentrations of this ligand may cause progressive cross-linking of membrane components to disrupt the recognition mechanisms required for lymphocyte entry into nodes. It is still uncertain whether this blockade of cell traffic reflects simple

MASKING OF MEMBRANE COMPONENTS OR SECONDARY METABOLIC CHANGES INDUCED BY THIS MITOGEN. ATTEMPTS TO RESOLVE THIS ISSUE ARE IN PROGRESS UTILIZING MONO AND DIVALENT FORMS OF CON A WHICH COMBINE WITH SURFACE OLIGOSACCHARIDES AND INDUCE BLASTOGENESIS WITHOUT CAUSING THE RECEPTOR CROSS-LINKAGE REQUIRED FOR PATCH FORMATION. RESULTS DESCRIBED IN PROJECT I HAVE SHOWN THAT PATCHES OF FERRITIN-CONJUGATED CON A PERSIST ON THE LYMPHOCYTE SURFACE FOR SEVERAL HOURS. SINCE OUR PRELIMINARY ULTRASTRUCTURAL STUDIES ON THE TRAFFIC OF SUCH CELLS IN NORMAL RECIPIENTS HAVE IDENTIFIED NUMEROUS LIGAND-BEARING CELLS IN HEV LUMENS WHILE ONLY OCCASIONAL CELLS EMIGRATE INTO THE HEV WALLS, IT SEEMS LIKELY THAT CON A INTERFERES WITH MECHANISMS RESPONSIBLE FOR THE SELECTIVE ATTACHMENT OF LYMPHOCYTES TO HEV SURFACE.

D) STUDIES WITH ANTI-IMMUNOGLOBULINS. TO ACHIEVE MAXIMAL REACTIVITY WITH THE VARIOUS LYMPHOCYTE POPULATION IN THORACIC DUCT LYMPH, RABBIT-ANTI-RAT IMMUNOGLOBULIN (CAPPELL LABORATORIES, DOWNINGTON, PA.) WAS USED WITHOUT PRIOR ABSORBTION WITH LEWIS THYMOCYTES TO REMOVE HETEROSPECIFIC ANTIBODIES. THIS PREPARATION WAS FOUND TO BE WEAKLY MITOGENIC IN LYMPHOCYTE CULTURES (TABLE 7) AND CAUSED SURFACE CAPPING IN 74% OF THE CELLS WITHOUT INDUCING AGGLUTINATION OR LYSIS WHEN THE ANTI IGG COATED LYMPHOCYTES WERE ADDED TO NORMAL RAT SERUM. RAT THORACIC DUCT CELLS PRE-INCUBATED WITH THIS ANTI IGG EXHIBITED NORMAL PATTERNS OF RANDOM AND DIRECTIONAL MIGRATION WHEN TESTED IN THE NELSON CHEMOTACTIC ASSAY. IN CONTRAST, RABBIT-ANTI-RAT THYMOCYTE GLOBULIN CAUSED COMPLETE LYSIS OF ALL THORACIC DUCT LYMPHOCYTE POPULATIONS WHEN SERUM WAS ADDED TO THE VARIOUS IN VITRO ASSAY PROCEDURES.

THE DIFFERENCES BETWEEN THESE ANTIBODY PREPARATIONS WAS EQUALLY APPARENT IN THE IN VIVO STUDIES OF TRAFFIC KINETICS (TABLE 8). RADIOLABELED LYMPHOCYTES PRE-INCUBATED WITH ATG FAILED TO ENTER LYMPHATIC TISSUES AND THIS WAS CLEARLY DUE TO LYSIS AFTER EXPOSURE TO COMPLEMENT IN VIVO AS THE LIVER WAS THE ONLY ORGAN SHOWING SIGNIFICANT RADIOACTIVITY IN THE RECIPIENTS. IN CONTRAST, CELLS EXPOSED TO ANTI IGG DISPLAYED PERFECTLY NORMAL PATTERNS OF UPTAKE AND REDISTRIBUTION IN RAT ORGANS DESPITE OUR FINDING THAT NEARLY 3/4 OF THESE CELLS CARRIED IMMUNOGLOBULIN LIGANDS COMPLEXED TO THEIR SURFACES. WHILE THE RESULTS DESCRIBED IN PROJECT I DEMONSTRATED THAT THE ANTI IGG COMPLEXES WERE SHED FROM LYMPHOCYTE SURFACES WITHIN 4 HOURS, IT IS KNOWN FROM STUDIES IN OTHER LABORATORIES THAT 12-20 HOURS ARE REQUIRED FOR LYMPHOCYTES TO REGENERATE THEIR SURFACE IGG RECEPTORS. TOGETHER, THESE OBSERVATIONS CLEARLY ESTABLISH THAT SURFACE IMMUNOGLOBULINS ARE NOT THE MEMBRANE COMPONENTS WHICH MEDIATE THE SURFACE RECOGNITION REQUIRED FOR LYMPHOCYTE HOMING INTO HEV.

PROJECT 3. THE SELECTIVE PHAGOCYTIC ACTIVITIES OF HIGH ENDOTHELIAL CELLS. (THE "SCRUBBING HYPOTHESIS").

CYTOCHEMICAL AND ULTRASTRUCTURAL OBSERVATIONS PRESENTED IN OUR PREVIOUS PROGRESS REPORTS HAVE ESTABLISHED THAT THE HIGH ENDOTHELIAL CELLS IN LYMPH NODE VENULES POSSESS STRUCTURAL AND METABOLIC PROPERTIES CONSISTENT WITH SPECIALIZED FUNCTIONS NOT FOUND IN OTHER TYPES OF VASCULAR ENDOTHELIUM. WE HAVE NOW COMPLETED A SERIES OF STUDIES COMBINING REGIONAL ARTERIAL PERFUSION OF DIFFERENT COLLOIDS, BACTERIA AND ALTERED CELLS WITH ULTRASTRUCTURAL STUDIES OF THE REGIONAL AND DISTANT NODES WHICH INDICATE THAT THIS ENDOTHELIUM CAN DISPLAY PHAGOCYTIC ACTIVITIES UNDER NEAR PHYSIOLOGIC CONDITIONS.

In ultrastructural studies of regional nodes draining skin allografts, pertussis vaccine and VEE vaccine in complete Freund's adjuvant, we have observed sporadic evidence for the phagocytosis of fibrin strands and cellular debris from the venular lumens by high endothelial cells. In this setting, endothelial cell phagocytic activity could have real relevance in clearing the vascular system of small clots and damaged cells appearing as histamine, kinins, activated complement components and lymphokines leak from the parenchyma of the inflamed node into HEV lumens. Further, we have demonstrated endocytosed carbon particles, thorium dioxide and ferritin granules admixed with membrane and lipid inclusions within lysosomes in high endothelial cells after infusing these inert particles into the regional circulation. While such observations are not truly quantitative, the absence of endocytosed particles in the endothelium lining other nodal vessels suggested that this phagocytic activity was confined to HEV. Following intra-arterial infusions with killed and washed pneumococci and staphylococci, there was no morphologic evidence for phagocytosis of these organisms by HEV despite the frequent finding of numerous bacteria lying free or enmeshed within fibrin in the vessel lumens. However, pre-incubating these bacteria in specific antibody, or the infusion of organisms known to bind C'_3 and activate the alternate complement pathway (S typhosa and yeast) yielded typical phagocytosis where the micro-organisms were engulfed by filopodia; packaged within phagolysosomes, and degraded within high endothelial cells. Similarly, regional infusions with allogeneic erythrocytes and lymphocytes failed to initiate phagocytic events in the nodal vessels, but identical experiments employing cells treated with specific allo-antibodies resulted in endocytosis of both whole cells and cellular fragments by the HEV endothelium. When combined, these results suggest that high endothelial cells are capable of "non-specific phagocytosis" and the highly selective, segmental phagocytosis dependent upon F-c and C'_3 receptors which Silverstein has described as the "zipper" effect. Experiments are in progress to evaluate this concept further using indicator bacteria to determine whether HEV can be shown to display F-c and C'_3 receptors on their luminal surfaces.

The most intriguing possibility raised by the phagocytic capacity of HEV is that this endothelium may remove ligands, adsorbed proteins or irrelevant antigens from lymphocyte surfaces just before these immunocompetent cells enter the nodal microenvironment where the critical sequence of antigen presentation and cellular collaboration occur. A series of ultrastructural studies made in collaboration with Major A. O. Anderson and Dr. John White at USAMRIID are underway evaluating this hypothesis. In the initial experiments syngeneic rat thoracic duct lymphocytes were incubated with peroxidase-conjugated rabbit-anti-rat IgG for 30 minutes at 37°C. As shown in Projects 1 and 2, this resulted in surface patching and capping of some 74% of the cells examined by electron microscopy. Aliquots of these cells were then washed and transfused into normal Lewis rats. Their axillary, submandibular and mesenteric nodes were excised 3-120 minutes later and processed for electron microscopy using the diamino-benzidine reaction to localize the peroxidase activity. In all of the nodes examined at 10-120 minutes post-infusion, numerous lymphocytes displaying patches and caps of electron-dense reaction product on their surfaces were found in the lumens and walls of HEV. As these cells emigrated across the venular wall, the peroxidase tracer material appeared to be stripped from their surfaces by focal membrane segments of adjacent endothelial cells where the subplasmalemmal

microfilaments invaginated the membrane and formed endobytic vacuoles containing reaction product. Since these emigrating lymphocytes retained their normal ultrastructural characteristics and showed no signs of injury, this selective phagocytosis appeared to selectively strip foreign material from their membranes (i.e. "scrubbing"). The similarities between this sequence of events and the selective, segmental phagocytosis described in macrophages (Silverstein et al, 1974-1976) is quite striking. However, it is widely recognized that many cells endocytose horseradish peroxidase non-specifically; so further studies were necessary to determine whether other electron-dense tracers could be endocytosed from lymphocyte surfaces. Experiments using ferritin-conjugated anti IgG as the surface ligand have shown an identical sequence of selective stripping of ferritin granules from the outer membranes of emigrating lymphocytes by adjacent high endothelial cells, so it seems quite unlikely that this phenomenon is an artifact resulting from non-specific endocytic mechanisms.

The physiologic relevance of "scrubbing" may be more difficult to establish as the results described in Project I have shown that anti-immunoglobulin-membrane complexes are rapidly cleared by spontaneous shedding in vitro without requiring interactions with another cell type. We are currently attempting to resolve this issue in ultrastructural studies of nodes from rats infused with lymphocytes pre-incubated in more stable surface ligands (ruthenium red, alcian blue and con A) as described earlier. Of particular interest here, are our preliminary observations on lymphocytes coated with ferritin-conjugated con A since data presented in Project I has shown that this ligand is slowly cleared from cell surfaces in vitro. A similar delayed clearance appears to hold for these cells in vivo as we have been able to demonstrate numerous con A bearing lymphocytes within HEV lumens for several hours post-infusion. While we have been unable to find clear-cut examples of selective removal of this tracer through endocytic activity of the high endothelial cells, this failure appears to result from the failure of most con A treated lymphocytes to attach to HEV surfaces and emigrate across the venular wall.

PROJECT 4. Cytoskeletal Controls of Lymphocyte Recirculation.

a) Studies with Cytochalasins. The fungal metabolites (cytochalasins) have attracted a great deal of attention in the past decade because of their effects upon membrane transport and motile processes in eukaryotic cells. At relatively low concentrations, cytochalasins B, D and E inhibit glucose, nucleoside and purine transport. At relatively high concentrations these agents inhibit various motile processes including cell locomotion, cytoplasmic streaming, cytokinesis and axonal growth cone activity. In attempts to minimize these diverse effects, we have utilized cytochalasin A in studies of lymphocyte traffic as this material purportedly causes longlasting suppression of cellular motility without altering transport mechanisms. The results of combined radiokinetic, autoradiographic and morphologic studies described in our last annual progress report demonstrated that in vitro exposure of rat thoracic duct lymphocytes to cytochalasin A at concentrations of 8-10 µg/ml caused microfilament aggregation, loss of microvilli and complete suppression of cellular motility which persisted for several hours after the cells were removed from the cytochalasin solution. Upon transfusion into syngeneic recipients, the treated lymphocytes adhered selectively to HEV surfaces, but their subsequent movement across the venular wall into the nodes was markedly

delayed. Since these observations provided the first experimental evidence that lymphocyte entry into peripheral nodes can be separated into an attachment phase mediated by selective membrane interactions which is followed by active migration of motile lymphocytes across HEV walls, we have undertaken further study of this phenomenon.

Dose response studies summarized in Table 9 showed that the viability of rat thoracic duct lymphocytes was unaltered by incubation with cytochalasin A at concentrations ranging from 0.2 to 10 ug/ml for ½ hour at 37°C. When the motility of normal lymphocytes was assayed in modified Nelson plates, a mean number of 91 cells were seen migrating beneath the agarose within a 66 μ wide strip connecting the centers of the inner-middle and outer wells after 12 hours of incubation. Direct measurements showed that randomly migrating cells within this population sample had covered a mean linear distance of 40 μ, while lymphocytes exhibiting directional migration towards endotoxin activated serum moved a mean distance of 186 μ. Cells treated with 0.2 μg/ml of cytochalasin A displayed virtually identical patterns of movement in the same assay system. Pre-incubation of the lymphocyte populations with cytochalasin A at dosages ranging from 2-4 μg/ml resulted in a progressive decline in the total number of migratory cells and the mean distance migrated. Treatment with cytochalasin A at concentrations of 6 or more ug/ml completely suppressed both random and directional lymphocyte migration for at least 12 hours in this assay system. Attempts to correlate this altered motility with ultrastructural changes defined by scanning and transmission electron microscopy are also shown in Table 9. In brief summary, these studies demonstrated progressive aggregation of the subplasmalemmal microfilament network into irregular clumps as the dose of cytochalasin A was increased from 0.2 to 6 μg/ml. This was paralleled by loss of microvilli (at 2-4 ug/ml) yielding cells with a smooth, swollen appearance interspersed with zeotic blebs (6-8 ug/ml). As these sequential, dose-related changes could all be explained by progressive focal aggregation of the microfilaments with concomitant separation of some filaments from intervening segments of the inner membrane, these observations provide further support for postulates that the cytochalasins disrupt cellular motility by combining with high affinity receptors on actin binding proteins to aggregate thin filaments. Previous suggestions that cytochalasin A might inhibit motile processes because of a unique ability to cross-link sulfhydryl groups on the membrane no longer seem tenable in light of recent evidence that this cross-linkage cannot occur under physiologic conditions (Staussel et al, 1976).

The relevance of these dose-dependent changes to <u>in vivo</u> lymphocyte traffic kinetics has been established in a series of radiokinetic studies using ^{3}H-uridine labeled lymphocytes as described in previous sections. The results shown in Table 10 show that pre-incubation of radiolabeled lymphocytes with cytochalasin at concentrations ranging from 4 to 10 μg/ml almost totally suppressed the entry of these cells into peripheral lymph nodes, and this effect persisted up to 24 hours after the cells had been removed from the cytochalasin solution. Similarly, cells exposed to 2 ug/ml demonstrated a 40-50% reduction in their traffic through lymphoid organs after transfusion into normal Lewis recipients. Since this drug treatment did not yield increased hepatic uptake of the radiolabel or alter cell viability by dye exclusion criteria, it seemed unlikely that these traffic changes could be attributed to direct cytotoxicity of cytochalasin A. Further, when these results were compared

WITH THE DATA IN TABLE 9, THERE WAS A CLOSE CORRELATION BETWEEN THE DOSES REQUIRED TO INHIBIT LYMPHOCYTE MIGRATION IN VITRO AND THOSE CAUSING COMPLETE SUPPRESSION OF LYMPHOCYTE RECIRCULATION IN VIVO.

CAUTION MUST BE TAKEN BEFORE GENERALIZING THE ABOVE FINDINGS TO ALL OF THE VARIOUS CYTOCHALASIN SUBTYPES. RESULTS PRESENTED IN TABLE 11 DEMONSTRATE THAT LYMPHOCYTES EXPOSED TO CYTOCHALASIN B AT 10 UG/ML EXHIBITED A 50-70% REDUCTION IN THEIR ABILITY TO RECIRCULATE THROUGH LYMPHOID ORGANS AFTER TRANSFUSION INTO NORMAL RATS. THIS OBSERVATION WAS UNEXPECTED SINCE MOST PUBLISHED REPORTS HAVE FOUND THAT THE MICROFILAMENT AGGREGATION INDUCED BY CYTOCHALASIN B WAS FULLY REVERSIBLE WHEN THE CELLS WERE WASHED FREE OF THE DRUG. WHILE IT IS POSSIBLE THAT IN VITRO TRAFFIC KINETICS MAY PROVIDE A MORE DEMANDING BIOLOGICAL TEST FOR ASSAYING RESIDUAL MICROFILAMENTOUS DAMAGE, EACH OF THE RECIPIENTS INFUSED WITH THESE TREATED CELLS SHOWED HEPATIC UPTAKE OF RADIOACTIVITY AT LEVELS 2-3X THAT SEEN USING CYTOCHALASIN A. AS CYTOCHALASIN B IS ALSO KNOWN TO DISRUPT MEMBRANE TRANSPORT FUNCTIONS IT IS LIKELY THAT THE IMPAIRED LYMPHOCYTE TRAFFIC PRODUCED BY THIS AGENT MAY RESULT FROM CELL DAMAGE UNRELATED TO THE MICROFILAMENT SYSTEM.

COMBINED AUTORADIOGRAPHIC AND ULTRASTRUCTURAL STUDIES DESCRIBED IN OUR LAST ANNUAL PROGRESS REPORT DEMONSTRATED THAT CYTOCHALASIN A TREATED LYMPHOCYTES COULD ATTACH TO HEV SURFACES, BUT THEIR SUBSEQUENT EMIGRATION ACROSS THE VENULAR WALL WAS IMPAIRED SO THAT MANY OF THESE VENULES APPEARED TO BE LINED BY A MONOLAYER OF ALTERED LYMPHOCYTES. FURTHER ANALYSIS OF THIS PHENOMENON BY TRANSMISSION ELECTRON MICROSCOPY HAS SHOWN THAT CYTOCHALASIN A TREATED LYMPHOCYTES ATTACH TO HEV THROUGH FOCAL MEMBRANE SEGMENTS MEASURING 3000°A IN DIAMETER WHICH WERE ALWAYS SITUATED DIRECTLY OVER LARGE AGGREGATES OF SUBPLASMALEMMAL MICROFILAMENTS. BENEATH INTERVENING SEGMENTS OF THE MEMBRANE, THE THIN FILAMENT NETWORK WAS EITHER ABSENT OR ATTENUATED. WHEN THESE CELLS MOVED ACROSS THE HEV WALL, THEIR CYTOPLASMIC MICROFILAMENTS APPEARED TO BE RETRACTED FROM THE USUAL SUB-MEMBRANOUS LOCATION AND CONCENTRATED WITHIN ECCENTRIC BUNDLES IN THE CONSTRICTION ZONE. SUCH FINDINGS SUGGEST THAT "RECEPTORS" REQUIRED FOR SURFACE ATTACHMENT MAY BE LINKED TO THE MICROFILAMENTOUS NETWORK AND CONCENTRATE INTO FOCAL PATCHES AS CYTOCHALASIN A INDUCED THEIR THIN FILAMENTS TO AGGREGATE. WHEN THE CELLS LOCOMOTE, SOME OF THESE DAMAGED THIN FILAMENTS MAY SEPARATE FROM THEIR PERIPHERAL ATTACHMENTS AND RETRACT INTO ASYMMETRIC BUNDLES AT SITES WHERE MEMBRANE ANCHORAGE IS MAINTAINED WITHIN THE CONSTRICTION ZONE. WHILE CONFIRMATION OF THIS HYPOTHESIS BY OTHER TECHNIQUES IS NEEDED, SUCH STRUCTURAL CHANGES CERTAINLY SEEM TO PROVIDE A PLAUSIBLE EXPLANATION FOR THE DELAYED MOVEMENTOF CYTOCHALASIN A TREATED LYMPHOCYTES ACROSS VENULAR WALLS. IN ADDITION, THESE ULTRASTRUCTURAL STUDIES HAVE SHOWN THAT BOTH EXTRACELLULAR AND SURFACE DEBRIS WERE STRIPPED FROM THE MEMBRANES OF THE TREATED LYMPHOCYTES AND ENDOCYTOSED BY SELECTIVE, SEGMENTAL PHAGOCYTIC ACTIVITY OF THE ADJACENT ENDOTHELIAL CELLS. THESE OBSERVATIONS APPEAR TO PROVIDE FURTHER SUPPORT FOR THE "SCRUBBING" CONCEPT DESCRIBED IN PROJECT 2.

B) STUDIES WITH COLCHICINE. WE HAVE COMPLETED A SERIES OF EXPERIMENTS ATTEMPTING TO DEFINE THE ROLE OF THE CYTOSKELETON IN REGULATING LYMPHOCYTE RECIRCULATION USING PHARMACOLOGIC TREATMENT WITH COLCHICINE TO DISRUPT THE LABILE MICROTUBULAR NETWORK. IN VITRO STUDIES DEMONSTRATED THAT RAT THORACIC DUCT LYMPHOCYTES INCUBATED IN COLCHICINE AT CONCENTRATIONS RANGING FROM 10^{-2} TO 10^{-8}M FOR TIME INTERVALS RANGING FROM 1-24 HOURS REMAINED VIABLE BY DYE EXCLUSION CRITERIA UNTIL THE DRUG DOSAGE EXCEEDED

10^{-3}M. MEASUREMENTS OF LYMPHOCYTE MOTILITY BY PHASE MICROSCOPY AND MIGRATION UNDER AGAROSE INDICATED THAT THESE CELLS DISPLAYED NORMAL PATTERNS OF LOCOMOTION UNTIL TOXIC DRUG LEVELS OF 10^{-3}M COLCHICINE WERE EMPLOYED (TABLE 12). WHEN THESE COLCHICINE-TREATED CELLS WERE EXAMINED BY SCANNING ELECTRON MICROSCOPY, THEY DISPLAYED PERFECTLY NORMAL SURFACE CONFORMATION WITH MULTIPLE VILLUS PROJECTIONS. WHILE WE HAVE THUS FAR BEEN UNABLE TO CONSISTENTLY DEMONSTRATE AGGREGATED OR DISSOCIATED MICROTUBULES IN THESE CELLS BY TRANSMISSION ELECTRON MICROSCOPY, THIS CAN PROBABLY BE ATTRIBUTED TO THE FACT THAT RAT THORACIC DUCT LYMPHOCYTES POSSESS A SCANT MICROTUBULAR NETWORK WHICH IS LARGELY OBSCURED BY CYTOPLASMIC RIBOSOMES MAKING IT DIFFICULT TO DEMONSTRATE THESE STRUCTURES EVEN WHEN SECTIONS ARE OBTAINED THROUGH THE CENTRIOLE OF NORMAL CELLS. CERTAINLY, THE INHIBITION OF MICROTUBULAR ASSEMBLY BY COLCHICINE HAS BEEN SUBSTANTIATED IN MOST OTHER CELL TYPES AND HAS BEEN GENERALLY ACCEPTED AS THE TYPICAL STRUCTURAL CHANGE INDUCED BY THIS DRUG. DESPITE THIS, OUR INITIAL SURVEY ON THE <u>IN VIVO</u> TRAFFIC OF RADIOLABELED LYMPHOCYTES PRE-INCUBATED WITH COLCHICINE SHOWED A NORMAL PATTERN OF ORGAN UPTAKE OF THESE CELLS AT 24 HOURS WHICH WAS ALTERED ONLY BY HIGH DRUG CONCENTRATIONS KNOWN TO CAUSE DIRECT CYTOTOXICITY (TABLE 12). HOWEVER, WHEN THE RECIRCULATION OF THESE DRUG TREATED CELLS WAS ANALYZED IN MORE DETAIL BY MONITORING THE ORGAN DISTRIBUTION OF RADIOACTIVITY AT EARLIER TIME INTERVALS (TABLE 13), THE RESULTS DEMONSTRATED THAT NON-TOXIC DOSAGES OF COLCHICINE AT THE CONCENTRATIONS KNOWN TO DISRUPT THE MICROTUBULAR NETWORK (10^{-4} - 10^{-6}M) CAUSED A 50-60% REDUCTION IN THE ACCUMULATION OF THE RADIOLABELED LYMPHOCYTES IN LYMPHATIC TISSUES WHICH PERSISTED FOR 8-12 HOURS. SINCE THIS WAS A TRANSIENT EFFECT WHICH CLEARED COMPLETELY WITHIN 24 HOURS WITHOUT BEING PARALLELED BY INCREASED HEPATIC RADIOACTIVITY, THESE FINDINGS APPEARED TO BE ENTIRELY CONSISTENT WITH COLCHICINE EFFECTS UPON MICROTUBULES WHICH ARE KNOWN TO BE FULLY REVERSIBLE WITH TIME. THIS CONCEPT HAS BEEN SUPPORTED DIRECTLY IN A SERIES OF SIMILAR EXPERIMENTS USING LUMICOLCHICINE- A COLCHICINE ANALOGUE WHICH PURPORTEDLY POSSESSES ALL OF THE PHARMACOLOGIC ACTIVITIES OF THE PARENT DRUG EXCEPT MICROTUBULAR TOXICITY. DATA PRESENTED IN TABLE 14 DEMONSTRATES THAT RADIOLABELED LYMPHOCYTES PRE-INCUBATED WITH 10^{-4} - 10^{-5}M LUMICOLCHICINE DISPLAYED PERFECTLY NORMAL PATTERNS OF ACCUMULATION WITHIN LYMPHOID ORGANS OVER TIME INTERVALS RANGING FROM ½ TO 24 HOURS AFTER TRANSFUSION INTO NORMAL RECIPIENTS. SUCH OBSERVATIONS PROVIDE THE BEST MEANS CURRENTLY AVAILABLE FOR PROVING THAT AN INTACT MICROTUBULAR NETWORK IS ESSENTIAL FOR MAINTAINING NORMAL PATTERNS OF LYMPHOCYTE RECIRCULATION <u>IN VIVO</u>. THE PRECISE FUNCTIONS OF THE MICROTUBULES IN THIS PROCESS ARE STILL UNCERTAIN SINCE THERE APPEARS TO BE GENERAL AGREEMENT THAT MOST CELLS CONTINUE TO DISPLAY NORMAL PATTERNS OF LOCOMOTION AND CHEMOTACTIC RESPONSES <u>IN VITRO</u> AFTER COLCHICINE TREATMENT. HOWEVER, SEQUENTIAL AUTORADIOGRAPHIC STUDIES ANALYZING THE RELATIVE RATES OF ENTRY, REDISTRIBUTION AND TRANSIT OF COLCHICINE-TREATED LYMPHOCYTES THROUGH LYMPH NODES <u>IN VIVO</u>, INDICATED THAT THIS CELL POPULATION WAS DEFICIENT IN ITS ABILITY TO ATTACH UPON HEV SURFACES, BUT THE FEW REMAINING CELLS WHICH COULD ENGAGE HEV CONTINUED TO MIGRATE THROUGH THE NODE IN A NORMAL MANNER (TABLE 15). AGAIN, THIS EFFECT APPEARED TO BE TRANSIENT AND AT LATER TIME INTERVALS (8-24 HOURS POST-DRUG TREATMENT) THE INFUSED CELLS SHOWED NORMAL DISTRIBUTION PATTERNS IN THE LYMPHOID ORGANS. WHEN COMBINED WITH THE RESULTS DESCRIBED EARLIER, THESE FINDINGS SUGGEST THAT THE MICROTUBULAR NETWORK MAY PLAY A CRITICAL ROLE IN ANCHORING RECEPTORS OR STABILIZING MEMBRANE SEGMENTS NECESSARY FOR THE SUCCESSFUL ATTACHMENT OF LYMPHOCYTES TO HEV SURFACES.

ADDITIONAL EXPERIMENTS HAVE BEEN MADE TO DETERMINE WHETHER SIMILAR EFFECTS COULD BE PRODUCED WHEN THE HOSTS WERE TREATED WITH COLCHICINE.

Results described in our previous annual progress report have shown that rats injected intraperitoneally with 1 mg/Kg colchicine displayed a mild lymphocytosis in the peripheral blood which was paralleled by an abrupt fall in the numbers of lymphocytes attached to HEV and a gradual decline in the hourly output of thoracic duct lymphocytes which combined to produce nodal hypocellularity. In the attempt to determine whether these altered traffic kinetics resulted from systemic drug toxicity or direct effects upon the recirculating lymphocytes, we collected thoracic duct lymphocytes from colchicine-treated and control rats. After labeling _in vitro_ with ^3H-uridine, both groups of cells were transfused back into syngeneic recipients and their traffic kinetics compared by whole organ counting. Results shown in Table 16 clearly indicated that the ability of the lymphocyte obtained from colchicine-treated rats to "home" into peripheral lymph nodes was markedly reduced (20-25% of the level seen with control cells). As both groups of cells displayed normal viability by dye exclusion criteria, this difference probably reflects non-lethal, pharmacologic alterations induced by colchicine effects in the host. However, we do not yet have an adequate explanation as to why these changes appear to persist for more than 24 hours in contrast to the short term, fully reversible alterations induced by treating lymphocytes with colchicine _in vitro_.

Data from complementary experiments analyzing the traffic of normal radiolabeled lymphocytes after their infusion into recipients treated with colchicine are shown in Table 17. The results demonstrate that the intraperitoneal injection of colchicine at ½ hour before and 8 hours after intravenous infusion with ^3H-uridine labeled cells almost totally suppressed the nodal uptake of radioactivity for intervals extending up to 24 hours. Further, these alterations were dose dependent with 1 mg/Kg causing an 85% decrease in nodal radioactivity while 0.1 mg/Kg induced less than 50% suppression. Since results described in our last progress report illustrated that a single injection with colchicine ½ - 2 hours before cell infusion inhibited lymphocyte traffic for 6-8 hours which was followed by complete recovery of normal recirculation patterns between 8-24 hours, these changes also appear to be reversible unless recovery is blocked by a second drug injection. Since pharmokinetics studies by other investigators have shown that colchicine is rapidly cleared from the blood but high tissue levels are achieved in the spleen and lymph nodes, it is possible that the traffic changes described above may represent the _in vivo_ correlate of the lymphocyte microtubular changes induced by _in vitro_ drug exposure. However, two lines of evidence argue against this simplistic explanation. First, the maximal blood and tissue levels achieved by treating rats with 1 - 0.1 mg/Kg colchicine are considerably less than the 10^{-4} - 10^{-5}M concentrations required to alter lymphocyte traffic by _in vitro_ incubation. Second, recent studies have shown that prolonging the interval between colchicine injection and cell infusion out to 3-5 hours still results in impaired traffic through lymphatic tissues despite a fall in the blood colchicine level to virtually undetectable values when the radiolabeled lymphocytes were introduced into the circulation. Such observations suggest that colchicine can alter cellular traffic _in vivo_ by mechanisms other than causing microtubular lesions in lymphocytes. This concept is supported by our recent ultrastructural observations that colchicine dosages at 1.0 - 0.1 mg/Kg caused a marked decrease in both the number and the peripheral distribution of the microtubules within high endothelial cells. Of particular interest, is our demonstration that microtubular networks in these cells lose their usual radiating pattern from the Golgi out to the membrane and become concentrated within irregular clumps near the centriole.

If these tubules normally contribute in stabilizing the outer membrane, it seems quite plausible that such changes could impair surface interactions with lymphocytes. Since data summarized above and in our last progress report indicates that the block of lymphocyte recirculation in colchicine-treated rats results from the failure of blood-borne lymphocytes to attach upon HEV surfaces, this structural alteration in the endothelial cell microtubular network is particularly interesting. Further testing of this hypothesis is now in progress employing other pharmacologic agents which share the common capacity to disrupt the cytoskeleton (vinca alkyloids and topical anesthetics). Preliminary observations with Vincristine (Table 18) indicate that relatively high doses of this drug (10^{-4} - 10^{-6}M) can also suppress lymphocyte traffic in vivo, and if these findings are supported by future studies there will be strong circumstantial evidence that the surface interactions required for "homing" are dependent upon cytoskeletal integrity in both the lymphocytes and the endothelial cells. Since there are a wide variety of agents which can influence these cytoplasmic structures, these results may reveal entirely new means for pharmacologic manipulation of immune responses in vivo.

PROJECT 5. Studies of Lymphocyte Chemotaxis.

Results described in our last annual progress report have shown that modifications of the in vitro techniques described by Nelson et al (1975) can be successfully used to demonstrate both random and directional migration of lymphocytes under agarose in a 3-chamber system devoid of extraneous serum products which provides stringent criteria for studying chemotaxis. Each plate was examined by light microscopy to determine the morphology of the migrating cells and an ocular grid system was employed to measure distances the cells had migrated from the edge of the center well. The number of migrating cells and the distances they had moved along a line connecting the centers of all 3 wells was counted and plotted by the stem-leaf method to provide estimates of both random and directional migration for a large population sample. Measurement of the mean linear distance covered by the farthest 10 cells migrating from the center well towards the chemotactic factor (A) and that found for similar cells on the opposite side facing control solutions (B) were also used to score the chemotactic index (A/B) and the chemotactic differential (A-B) defined by Nelson.

Studies comparing random and directional migration responses of the mixed cellular populations present in thoracic duct lymph to endotoxin activated serum (EAS) in the outer well (A) and normal rat serum in the inner well (B) are shown in Table 19. The results demonstrated that both granulocytes and macrophages rapidly migrated out from the center well during the initial 2-7 hours of incubation. Further, both cell types consistently showed greater movement towards wells containing EAS than was seen on the opposite side facing control sera. This appeared to reflect true chemotactic responses and not the non-specific stimulation of random cellular locomotion as the addition of progressive dilutions of EAS into the center well at the start of each run either totally suppressed or markedly decreased outward cellular migration. The small lymphocytes residing within thoracic duct lymph exhibited more delayed and slower patterns of migration, but after 12-18 hours of culture these cells showed clear evidence of directional movement (mean chemotactic index of 4.3 and a mean chemotactic differential of 132 microns) with a definite shift in the population distribution curve towards the EAS-containing well. Since this response was ablated by mixing EAS with lymphocytes in the center well, it appeared to represent true chemotaxis (not chemokinesis). Because of reports indicating that the expression of surface receptors can

vary between lymphocytes in different stages of maturation, additional experiments were made to analyze the chemotactic responses of lymphocytes transformed into the "blast cell" state by mitogen stimulation. In one series of studies, both rat spleen cells and thoracic duct lymphocytes were cultured with 1 μg/ml of concanavalin A (a selective T-cell mitogen). At 48-72 hours, direct cell counts and histological examination of stained smears showed that virtually all (88-94%) of the surviving cells displayed morphologic characteristics typical of blast cells. When these T-blasts were placed into the center wells of the agarose plates, they exhibited early and rapid migration with many cells covering several hundred microns of linear distance within 3-4 hours. Assay of the response of these cells to EAS showed a dramatic shift in the population distribution curve towards the A well with a chemotactic index of 5.4 and a mean chemotactic differential of 1130 microns. Equally interesting was the finding of typical mitolic figures in many cells which had migrated considerable distances from the center well. Identical observations were then made using B-blasts generated by culturing rat spleen cells with 10-50 ug/ml of LPS for 48-72 hours. As this yielded a mixed cellular population containing approximately 40% B-blasts, 30% small lymphocytes and 30% macrophages, the identity of the migrating cells in the chemotactic assays could not be established by indirect phase microscopy, so that all cell counts were made by light microscopic study of fixed and stained coverslips removed from the agarose plates at the end of each run. Despite this limitation, repeated study of these cells has shown that only macrophages displayed consistent chemotactic responses in the Nelson assay. The B-blasts appeared to be far less motile than their T-cell counterparts and migrated only 100-200 μ in 12-18 hours. Further, they exhibited a mean chemotactic index of 1.4 and a mean chemotactic differential of 21 microns which probably indicate that the B-blasts do not respond with true chemotaxis along gradients of EAS. This finding was particularly interesting since similar studies with Lymphoma 8 cells (an undifferentiated B-cell rat lymphoma) isolated from the peripheral blood of Lewis rats during the leukosarcoma phase demonstrated that these neoplastic B-cells moved rapidly under agarose to travel distances of several hundred microns in 3-4 hours, but this migration was purely random with no evidence for a directional response to EAS. When all of these results are combined, they suggest that granulocytes, macrophages, small T-cells and T-blasts show chemotactic responses to a serum product within EAS while equally motile B-cells, B-blasts and their malignant counterparts may lack or fail to express the surface receptors for this chemoattractant factor. It is still uncertain whether the striking differences in the magnitude of the TDL and T-blast responses to EAS are related solely to differences in their rates of locomotion or reflect other changes such as the density of surface receptors. In our initial attempts to clarify this issue, Con A was added directly to the small lymphocyte population in the center well and the plates were cultured for sequential time intervals before adding EAS to the outer wells. To our surprise, the results (Table 20) suggested that Con A might actually suppress both random and directional migration over the first 36 hours of culture, but once blast cell transformation appeared these cells exhibited typical patterns of rapid random migration and chemotactic responses to EAS. Future studies with this model should be particularly useful in defining the metabolic and the structural changes induced by lectins which are responsible for the augmented <u>in vitro</u> chemotactic responses.

Because of the above findings we have wondered whether the delayed and rather slow locomotion of normal small lymphocytes in the agarose assay could be enhanced by metabolic perturbation of the cell. Since there

HAVE BEEN REPORTS THAT THE RANDOM MOVEMENT OF GRANULOCYTES, MACROPHAGES AND LYMPHOCYTES WAS INHIBITED BY EXOGENOUS CYCLIC AMP AND ENHANCED BY INCREASING INTRACELLULAR LEVELS OF CYCLIC GMP, WE HAVE UTILIZED SEVERAL DIFFERENT DRUGS IN ATTEMPTS TO MODIFY LYMPHOCYTE LOCOMOTION. HOWEVER, EXPERIMENTS EMPLOYING A RELATIVELY WIDE DOSAGE RANGE OF DIBUTYRL cGMP, ACETYLCHOLINE AND CARBAMYL CHOLINE HAVE ALL FAILED TO ENHANCE EITHER RANDOM OR DIRECTIONAL MIGRATION OF SMALL LYMPHOCYTES IN THE AGAROSE PLATES. TESTS USING DIBUTYRL cAMP, THEOPHYLLINE AND CHOLERA ENTEROTOXIN HAVE SUGGESTED THAT THESE AGENTS CAN SUPPRESS THE MOVEMENT OF BOTH NORMAL LYMPHOCYTES AND T-BLASTS IN VITRO, BUT THIS EFFECT HAS BEEN TOO SPORADIC TO PERMIT ADEQUATE QUANTITATION WITH THE TECHNIQUES AVAILABLE. INDEED, THE ONLY AGENT THAT WE HAVE FOUND WHICH CAN ENHANCE CHEMOTACTIC RESPONSES IS SERUM FROM LYMPHOMA 8 BEARING RATS. WHEN EITHER CRUDE SERUM OR THE GLOBULIN FRACTION OBTAINED BY ETHANOL PRECIPITATION WAS ADDED TO NORMAL RAT THORACIC DUCT LYMPHOCYTES IT INCREASED THEIR CHEMOTACTIC RESPONSES TO ENDOTOXIN ACTIVATED SERUM 2-4 FOLD WITHOUT ALTERING RANDOM MIGRATION (MEAN CHEMOTACTIC INDEX OF 7.4 AND A MEAN CHEMOTACTIC DIFFERENTIAL OF 502 MICRONS). AS THIS ENHANCING EFFECT COULD NOT BE DUPLICATED BY NORMAL SERUM, RAT GAMMA GLOBULIN OR RABBIT-ANTI-RAT IMMUNOGLOBULIN, THIS ACTIVITY APPEARS TO BE UNIQUE TO THE LYMPHOMA SYSTEM AND STUDIES ARE IN PROGRESS TO DETERMINE IF THE ACTIVE FACTOR IS RELATED TO POLYCLONAL ISOANTIBODIES OR CIRCULATING ANTIGEN-ANTIBODY COMPLEXES WHICH ARE KNOWN TO APPEAR IN SOME TRANSPLANTABLE ANIMAL LYMPHOMAS.

HAVING ESTABLISHED THAT NORMAL RAT SERUM EXHIBITS CHEMOATTRACTANT ACTIVITIES FOR ALL TYPES OF LEUKOCYTES (INCLUDING T-LYMPHOCYTES) AFTER ACTIVATION BY ENDOTOXIN, WE HAVE ENGAGED IN A LENGTHY SERIES OF STUDIES TO DETERMINE IF THESE EFFECTS ARE DUE TO SPLIT PRODUCTS FROM THE COMPLEMENT CASCADE WHICH ARE KNOWN TO BE CHEMOTACTIC IN OTHER IN VITRO ASSAY SYSTEMS. DATA SUMMARIZED IN TABLE 21 DEMONSTRATE THAT GRANULOCYTES, MACROPHAGES, TDL AND T-BLASTS ALL EXHIBIT CHEMOTACTIC RESPONSES TO ENDOTOXIN-ACTIVATED SERUM. SINCE THIS ACTIVITY IS COMPLETELY LOST BY PREHEATING THE SERUM AT 56° FOR 20 MINUTES BEFORE ADDING THE ENDOTOXIN, BUT REMAINS UNALTERED IF THE HEATING IS CARRIED OUT AFTER ENDOTOXIN TREATMENT, THESE RESULTS APPEAR ENTIRELY CONSISTENT WITH THE CHEMOATTRACTANT ACTIVITY BEING MEDIATED BY LOW MOLECULAR WEIGHT SPLIT PRODUCTS FROM THE COMPLEMENT SEQUENCE (C'_{3A} OR C'_{5A}). HOWEVER, THIS CONCEPT CANNOT EXPLAIN THE SURPRISING FINDINGS OBSERVED USING SERUM FROM RATS PRETREATED WITH COBRA VENOM AT DOSAGES OF 2 μG/GRM BODY WEIGHT. AT INTERVALS RANGING FROM 1-24 HOURS AFTER INJECTING PURIFIED COBRA VENOM FACTOR, THE SERUM WAS COMPLETELY DEVOID OF C'_3 - C'_9 ACTIVITIES DETECTABLE BY IN VITRO ASSAYS. WHEN THIS C' DEPLETED SERUM WAS ACTIVATED BY ENDOTOXIN AND TESTED AGAINST GRANULOCYTES AND MACROPHAGES IN THE NELSON ASSAY, THERE WAS NO SIGN OF DIRECTIONAL MIGRATION - A FINDING ENTIRELY CONSISTENT WITH DEPLETION OF THE C'_{3A} AND C'_{5A} CHEMOTACTIC FACTORS. DESPITE THIS, ALIQUOTS OF THE SAME SERUM SAMPLES CONSISTENTLY DISPLAYED CHEMOATTRACTANT ACTIVITIES FOR T-BLASTS AND TDL WHICH WERE COMPARABLE TO THE BEST RESPONSES SEEN AFTER ACTIVATION OF NORMAL SERUM. TOGETHER, THESE RESULTS PROVIDE THE FIRST EVILENCE FOR A SPECIFIC CHEMOATTRACTANT OF LYMPHOCYTES IN ENDOTOXIN-ACTIVATED SERUM WHICH MUST BE DISTINCT FROM C'_{3A} AND C'_{5A}. ATTEMPTS ARE NOW IN PROGRESS TO ISOLATE, CONCENTRATE AND CHARACTERIZE THIS FACTOR AS IT MAY WELL REPRESENT THE PROTOTYPE FOR STUDYING CHEMOTACTIC REGULATION OF LYMPHOCYTE TRAFFIC IN VIVO. WHILE THE ABOVE OBSERVATIONS DO NOT PRECLUDE THE POSSIBILITY THAT C' SPLIT PRODUCTS MAY ALSO PRODUCE NON-SELECTIVE CHEMOTACTIC EFFECTS UPON LYMPHOCYTES, IT IS MORE DIFFICULT TO PROPOSE A PHYSIOLOGIC FUNCTION FOR THIS WHICH WOULD BE CONSONANT WITH KNOWN CELLULAR RESPONSES TO INFLAMMATION IN VIVO. INDEED, THE POSSIBLE FUNCTION OF COMPLEMENT COMPONENTS AS DETERMINANTS OF LYMPHOCYTE RECIRCULATION

SEEM QUITE REMOTE AS THE DATA PRESENTED IN TABLE 22 CLEARLY DEMONSTRATE THAT RADIOLABELED LYMPHOCYTES MOVE THROUGH LYMPHOID ORGANS OF VENOM-TREATED RATS IN A PERFECTLY NORMAL FASHION DESPITE SUSTAINED SUPPRESSION OF THEIR $C'3$ - $C'9$ TO UNDETECTABLE LEVELS.

THE ABOVE RESULTS HAVE ALSO LED US TO ANALYZE OTHER WELL KNOWN CHEMOTACTIC AGENTS FOR POSSIBLE CHEMOATTRACTANT EFFECTS FOR LYMPHOCYTES IN THE MODIFIED NELSON PLATES. DATA SUMMARIZED IN TABLE 23 DEMONSTRATE THE RAT GRANULOCYTES ISOLATED FROM THE PERIPHERAL BLOOD BY FICOLL-HYPAQUE GRADIENT SEPARATION EXHIBITED TYPICAL DIRECTIONAL MIGRATION AGAINST CRUDE E. COLI CULTURE FILTRATE; THE CHEMOTACTIC TRIPEPTIDE-E-MET-LEU-PHE (SUPPLIED BY DR. ELLIOT SCHIFFMAN AT THE NATIONAL INSTITUTES OF HEALTH) AND THE CRUDE BASOPHILE CHEMOTACTIC FACTOR PREPARED FROM SUPERNATANTS OF RAT BUFFY COAT CELLS TREATED WITH THE CALCIUM IONOPHORE A23187 AS DESCRIBED BY LICHTENSTEIN. RAT PERITONEAL MACROPHAGES SHOWED SIMILAR, BUT SLIGHTLY LESS DRAMATIC RESPONSES TO EACH OF THESE AGENTS. IN CONTRAST, REPEATED STUDIES WITH THORACIC DUCT LYMPHOCYTES, T-BLASTS, B-BLASTS AND LYMPHOMA 8 CELLS ALL FAILED TO REVEAL ANY EVIDENCE FOR DIRECTIONAL MIGRATION OF DEFINED LYMPHOCYTE POPULATIONS TO AGENTS WHICH WERE CLEARLY POTENT STIMULATORS OF CHEMOTAXIS IN THE OTHER CELL LINES. WHEN COMBINED WITH THE OBSERVATIONS DESCRIBED EARLIER, THESE RESULTS STRONGLY SUGGEST THAT LYMPHOCYTES MUST HAVE COMPLETELY DIFFERENT TYPES OF SURFACE RECEPTOR MECHANISMS FOR REGULATING THEIR LOCOMOTION. INDEED, THESE FINDINGS WERE QUITE COMFORTING IN LIGHT OF OUR CURRENT KNOWLEDGE OF CELL TRAFFIC IN VIVO AS IT WOULD BE DIFFICULT TO EXPLAIN HOW LYMPHOCYTE MOVEMENT DIRECTED TOWARDS BACTERIAL PRODUCTS WOULD PROVIDE BIOLOGICAL ADVANTAGES FOR THE HOST.

ONE OF THE UNIQUE ADVANTAGES PROVIDED BY THE AGAROSE ASSAY IS THAT CELLS CAN BE PLATED INTO THE PERIPHERAL WELLS AND THEN CULTURED FOR SEVERAL HOURS TO 3 DAYS TO DETERMINE WHETHER THEY RELEASE FACTORS WHICH ARE CHEMOTACTIC FOR OTHER CELL TYPES ADDED TO THE CENTER WELL. OUR PRELIMINARY OBSERVATIONS USING THIS GENERAL EXPERIMENTAL DESIGN ARE LISTED IN TABLE 24. IN SUMMARY, THESE RESULTS PROVIDE THE FIRST EVIDENCE INDICATING THAT B-BLASTS PRODUCE FACTORS WHICH ARE CHEMOTACTIC FOR T-BLASTS BUT APPARENTLY LACK THIS EFFECT ON OTHER TYPES OF LYMPHOCYTES. SINCE THERE IS IN VIVO EVIDENCE SUGGESTING THAT SOME T-BLASTS MAY MOVE INTO GERMINAL CENTERS AND THE SURROUNDING MANTLE ZONE (B-CELL AREAS), THESE OBSERVATIONS MAY PROVIDE OUR FIRST INSIGHT INTO THE MECHANISMS WHICH ACCOUNT FOR THE REDISTRIBUTION OF LYMPHOCYTES INTO DIFFERENT ZONES OF LYMPHOID TISSUES. FURTHER STUDY OF THIS PHENOMENON AND THE POSSIBLE MEDIATORS ARE NOW IN PROGRESS.

PROJECT 6. THE MECHANISMS OF IMMUNOPOTENTIATION BY STANDARD ADJUVANTS.

EVIDENCE SUMMARIZED IN OUR PREVIOUS PROGRESS REPORTS AND CURRENT CONTRACT RENEWAL INDICATES THAT NON-SPECIFIC ADJUVANTS MAY POTENTIATE IMMUNE RESPONSES BY A VARIETY OF MECHANISMS, I.E.: DEPOT FORMATION; STIMULATION OF MACROPHAGES; GRANULOMA FORMATION; RECRUITING BOTH ANTIGEN-REACTIVE AND HELPER T-CELLS INTO THE NODE; INITIATING RELEASE OF LYMPHOKINES AND OTHER CELL PRODUCTS WHICH FACILITATE HELPER EFFECTS; ACTIVATING MACROPHAGES; STIMULATING T AND B CELLS DIRECTLY; ENHANCING THE PRODUCTION AND RELEASE OF HELPER T-CELLS FROM THE THYMUS, AND ETC. SINCE COMPLETE FREUND'S ADJUVANT (CFA) HAS BECOME GENERALLY ACCEPTED AS THE BEST PROTOTYPE FOR NON-SPECIFICALLY AUGMENTING IMMUNE RESPONSES IN WEAK IMMUNOGENS IN EXPERIMENTAL ANIMALS, WE HAVE INITIATED MAJOR EFFORTS TO DEFINE THE MECHANISMS OF ACTION OF THIS AGENT TO ACHIEVE THE NECESSARY BACKGROUND FOR OUR PROPOSED USE OF MURAMYL DIPEPTIDE AND CELL

PRODUCTS AS ADJUVANTS.

In these studies over 200 Lewis rats have been immunized with VEE vaccine in CFA. These animals were bled at sequential intervals to determine antibody titers by plaque neutralization assays and representative rats were killed at 1, 3, 7, 14, 28 and 56 days post-immunization to document microvascular, morphologic and cell traffic changes in their lymphatic tissues. Results shown in Table 25 demonstrate that subcutaneous injections of VEE in CFA caused a doubling in regional lymph node weight within 3-7 days. This was followed by a gradual decline to a mean of 35 mg which was maintained from 28 to 56 days post challenge. These changes were virtually identical to those seen using brief antigenic exposure to skin allografts despite previous postulates that CFA serves as a depot promoting long term exposure of the regional node to antigens. Equally interesting were observations that the total lymph node mass of rats injected with CFA gradually increased from a mean weight of approximately 850 mg to 1190 mg (Table 26) over the 8 week study interval while that seen in saline injected controls fluctuated between 750-800 mg. While this may represent responses to granuloma dissemination throughout the lymphoid system, this may not be the sole explanation as spleen weights in the CFA-treated rats remained unchanged over the same 56 day interval.

Morphometric techniques were employed to relate this regional nodal enlargement to changes in the mass of T and B cell zones. Results illustrated in Table 27 demonstrate that local CFA injections caused a 3-fold increase in mean cortical weight which persisted from 3 to 28 days post-challenge. After the initial inflammation resolved, this was paralleled by a 2-3x increase in both the number and estimated mass of germinal centers within the enlarged cortex. As the medullary cords became longer and more prominent from 7-28 days, there seemed to be little doubt that this immunization evoked intense cellular proliferation and differentiation in the B-cell zones consistent with a vigorous humoral antibody response. The sequential morphologic changes appearing within these nodes provided further insight into the possible mechanisms of immunopotentiation by CFA. Histologic scoring of the lymphocyte migration index (number of migrating lymphocytes per 100 high endothelial cells) strongly suggested that the traffic of blood-borne lymphocytes into the nodes was increased from 1 to 28 days post challenge. While autoradiographic studies monitoring the intranodal traffic of these cells are still pending, the frequent finding of loosely adherent clumps of lymphocytes with apparent "plugging" of the intermediate sinuses certainly supported increased traffic across the node with possible partial blockade of egress. While numerous activated macrophages were found in all sinus passages, typical granulomas first appeared in the outer nodal cortex at 14 days and gradually enlarged over the subsequent study interval. While it seems quite plausible that these changes may be related to altered antigen clearance from the node, this concept is just now being tested with labeled VEE. In any event, this combination of inflammation and macrophage activation appears to cause prolonged recruitment of recirculating lymphocytes through the regional node. Whole organ counts after infusing 3×10^8 radiolabeled thoracic duct cells into these hosts demonstrated that the 24 hour accumulation of radioactivity in the non-stimulated, contralateral nodes consistently ranged near 0.3% of the total injected dose over intervals ranging from 0 to 56 days. In contrast, the stimulated regional nodes consistently showed $1\frac{1}{2}$ - 3x greater uptake of radiolabel between 1 and 28 days post-CFA injection. While this clearly proves increased cellular traffic through the regional nodes, this

NON-SPECIFIC RECRUITMENT MAY BE RELATED IN PART TO AUGMENTED BLOOD FLOW THROUGH THE ENLARGED NODE SINCE THE SPECIFIC ACTIVITY (DPM PER GRAM OF TISSUE) WAS NOT SIGNIFICANTLY DIFFERENT IN THE REGIONAL AND CONTRALATERAL NODES AT EACH TIME INTERVAL STUDIED. AS LYMPHOCYTE RECIRCULATION THROUGH OTHER LYMPHOID ORGANS IN THE SAME HOSTS WAS UNALTERED (TABLE 30), IT SEEMED UNLIKELY THAT THE ENHANCED REGIONAL NODE TRAFFIC COULD BE ATTRIBUTED TO POSSIBLE SYSTEMIC CHANGES (I.E., NON-SPECIFIC STRESS, ETC.) PRODUCED BY CFA.

THERE IS FURTHER EVIDENCE SUGGESTING THAT THESE CELL TRAFFIC CHANGES ARE RELEVANT TO IMMUNOPOTENTIATION BY CFA. DATA COMPARING PLAQUE NEUTRALIZATION TITERS IN SERA FROM RATS IMMUNIZED VEE OR VEE IN CFA ARE SHOWN IN TABLE 31. THE RESULTS DEMONSTRATE THAT CFA INCREASES VEE ANTIBODY RESPONSES BY 10-15X, YIELDING EFFECTIVE PROTECTION AGAINST CHALLENGE WITH VIABLE ORGANISMS FOR TIME INTERVALS EXTENDING BEYOND 16 WEEKS. OF EVEN GREATER INTEREST ARE THE OBSERVATIONS THAT THYMECTOMY 10-14 DAYS BEFORE IMMUNIZATION, PROMOTED HIGHER AND MORE PROLONGED ANTIBODY TITERS REGARDLESS AS TO WHETHER THE RATS WERE INJECTED WITH VEE ALONE OR IN COMBINATION WITH CFA. THESE EFFECTS WERE CLEARLY DEPENDENT UPON THE REGIONAL NODE AND COULD NOT BE DUPLICATED BY INJECTING THE ANTIGEN-ADJUVANT MIXTURE INTO THE THYMUS WHICH HAS AN ATRETIC LYMPHATIC NETWORK. SIMILARLY, SURFACE-ACTIVE AGENTS LIKE DIGITONIN PRODUCED LOCAL INFLAMMATION AT THE ANTIGEN INJECTION SITES WHICH FAILED TO AUGMENT AND MAY EVEN HAVE SUPPRESSED IMMUNE RESPONSES TO VEE. SINCE THE VACCINE PREPARATION EMPLOYED IN THESE STUDIES APPEARED TO BE A POTENT IMMUNOGEN CAPABLE OF ELICITING ANTIBODY RESPONSES IN ALL RATS CHALLENGED, THESE RESULTS SUGGEST THAT ADULT RATS HAVE ADEQUATE TISSUE STORES OF THE IMMUNOCOMPETENT CELLS AND HELPER CELLS NEEDED TO MOUNT IMMUNE RESPONSES TO VEE ANTIGENS. THE CELL TRAFFIC CHANGES INDUCED BY CFA PROVIDE ONE OBVIOUS MECHANISM WHEREBY THESE CELLS MAY BE RECRUITED INTO REGIONAL NODES WHERE ANTIGEN IS SEQUESTERED. HOWEVER, THE FACT THAT THESE ANTIBODY RESPONSES CAN BE AUGMENTED AND PROLONGED BY THYMECTOMY, INDICATES THAT BOTH ANTIGEN-INDUCED AND ADJUVANT-MODIFIED IMMUNITY MAY BE MODULATED BY SUPPRESSOR CELLS, NEWLY FORMED AND RELEASED FROM THE THYMUS. IF CORRECT, THIS MAY PROVIDE A NEW PROTOTYPE FOR POTENTIATING IMMUNITY WHICH MAY BE OF CLINICAL RELEVANCE AS PHARMACOLOGIC MEANS ARE NOW AVAILABLE TO SELECTIVELY INHIBIT CELLULAR PROLIFERATION AND DIFFERENTIATION WITHIN THE THYMUS. FURTHER STUDIES OF THIS PHENOMENON ARE NOW IN PROGRESS.

PRESENTATIONS

1. Anderson, A.O. and Anderson, N.D.: Cellular interactions which regulate lymphocyte recirculation. (Presented at International Congress of Immunology, Sidney, Australia, in July 1977).

2. Anderson, N.D.: The biology of lymphocyte recirculation. (Presented at the College of William and Mary, in November 1977).

3. Anderson, N.D., Anderson, A.O., Hoffman, R.D., and White, J.D.: Cytoskeletal control of Lymphocyte recirculation. Studies with colchicine. (To be presented at FASEB meeting in April 1978, at Atlantic City, N.J.).

4. Anderson, A.O., Anderson, N.D., Hoffman, R.D., and White, J.D.: Cytoskeletal control of lymphocyte recirculation. Studies with cytochalasins. (To be presented at FASEB meeting in April, 1978 at Atlantic City, N.J.).

PUBLICATIONS

1. Bell, W.R., Anderson, N.D., and Anderson, A.O.: "Heparin-induced coagulopathy", J. Lab. Clin. Med. 89:741, 1977.

2. Anderson, N.D., Wyllie, R.G., and Shaker, I.J.: "The pathogenesis of endothelial injury in rejecting rat renal allografts", Johns Hopkins Medical Journal 141:135, 1977.

3. Anderson, N.D., Anderson, A.O., Hoffman, R.D., and White, J.D.: "Cytoskeletal control of lymphocyte recirculation. Studies with colchicine". Fed. Proc. 37:591, 1978.

4. Anderson, A.O., Anderson, N.D., Hoffman, R.D., and White, J.D.: "Cytoskeletal control of lymphocyte recirculation. Studies with the cytochalasins." Fed. Proc. 37:591, 1978.

5. Anderson, N.D., Wyllie, R.G., Frost, J., and Shaker, I.J.: "The pathology of unmodified rejection in rat kidneys grafted across major and minor histocompatibility loci." Johns Hopkins Medical Journal. (Submitted for publication).

6. Anderson, N.D., Colyer, R.J., and Riley, L.: "Skeletal changes during prolonged external irradiation: Alterations in the marrow, growth plate and osteoclast populations." J. Cell and Tissue Kinetics. (Submitted for publication).

LITERATURE CITED

(1) Feldman, M.J. Exp. Med. 163:737, 1972.

(2) Allison, A.C. Immunopotentiation. Ciba Foundation Symposium, pp. 73 and 96. Associated Scientific Publishers, New York, 1973.

(3) Dresser, D.E. and Phillips, J. Immunopotentiation. Ciba Foundation Symposium. pp. 3-27. Associated Scientific Publishers, New York, 1973.

(4) Gowans, J.L. Int. Rev. Expt. Path. 5:1, 1966.

(5) Ford, W.L. Cell. Tiss. Kinet. 2:171, 1969.

(6) Ford, W.L. and Gowans, J.L. Proc. Roy. Soc. B 168:24, 1967.

(7) Mitchell, J. Immunology 24:93, 1973.

(8) Zatz, M.M. J. Immunol. 111:706, 1973.

(9) Balfour, B.M. Immunology 8:230, 1965.

(10) Goldschneider, I. and McGregor, D.D. J. Exp. Med. 127:155, 1968.

(11) Sordat, B., Hess, M.W. and Cottier, H. Immunology 20:115, 1971.

(12) Anderson, A.O. and Anderson, N.D. Amer. J. Path. 80:387, 1975.

(13) Anderson, N.D., Anderson, A.O. and Wyllie, R.G. Amer. J. Path. 81:131, 1975.

(14) Atherton, A. and Born, G.V.R. J. Physiol. 222:447, 1972.

(15) Gesner, J.B. and Woodruff, J.J. Science 161:176, 1968.

(16) Marchesi, V.T. and Gowans, J.L. Proc. Roy. Soc. B. 159:283, 1964.

(17) Farr, A.G. and DeBruyn, P.P.H. Amer. J. Anat. 143:59, 1975.

(18) Henney, C. Personal Communication. 1976.

(19) Van Ewijk, W., Brons, N.H.C. and Rozing, J. Cell. Immunol. 19:245, 1975.

(20) Ward, P.A., Offen, C.A. and Montgomery, J.R. Fed. Proc. 30:1721, 1971.

(21) Keller, H.U., Hess, M.W. and Cottier, H. Antibiot. & Chemother. 19:112, 1974.

(22) Higuchi, Y., Honda, M. and Hayashi, H. Cell. Immunol. 15:100, 1975.

(23) Nelson, R.D., Quie, P.G. and Simmons, R.L. J. Immunol. 115:1650, 1975.

(24) Jarvis, S.C., Snyderman, R. and Cohen, H.J. Blood 48:717, 1976.

(25) Hay, J.B. and Hobbs, B.B. J. Exp. Med. 145:31, 1977.

(26) White, R.G. Antibiot. & Chemother. 19:382, 1974.

(27) Cahill, R.N.P., Frost, H. and Trinker, Z. J. Exp. Med. 143:70, 1976.

(24) Jarvis, S.C., [illegible], F. and [illegible], M.J. Blood [illegible], 1976.

(25) May, J.D. and [illegible], B.D. J. Exp. Med. [illegible], 1973.

(26) [illegible], R.G. [illegible] Z-Chemother. [illegible], 1976.

(27) [illegible], R.H.P., Frost, [illegible] and [illegible], [illegible] J. Exp. [illegible], 1976.

TABLE I

THE EFFECTS OF VARYING CONCENTRATIONS OF RUTHENIUM RED DYE UPON IN VITRO LYMPHOCYTE FUNCTIONS

Dose (mg/ml)	Mitogenic# Response	Agglutination	Viability by Trypan Blue	Surface Patches	Caps	Migration under Agarose* Random	Directional
1.0	140 cpm	+	18.2	-	-	-	-
0.5	186 cpm	±	96.8	100%	43%	+	+
0.1	612 cpm	0	97.2	98%	48%	+	+
Control	345 cpm	0	96.4	0	0	+	+

5×10^6 spleen cells cultured for 72 hours with dye and pulsed with ^3H-thymidine for 2 hours.

*Random and directional migration scored by migration under agarose in the modified Nelson chemotactic assay using endotoxin-activated serum.

TABLE 2

THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS AFTER INFUSING 300×10^6 ^{3}H-URIDINE LABELED LYMPHOCYTES TREATED WITH RUTHENIUM RED DYE

TIME AFTER CELL INFUSION	% TOTAL DOSE ACCUMULATING IN RAT ORGANS AFTER TREATING TDL WITH 1 MG RUTHENIUM RED#				
	LN	SPLEEN	LIVER	LUNGS	GUT
24 HR	1.44	1.51	14.30	0.68	2.14
	% TOTAL DOSE ACCUMULATING IN RAT ORGANS AFTER TREATING TDL WITH 0.05 MG RUTHENIUM RED				
	LN	SPLEEN	LIVER	LUNGS	GUT
2 HR	3.2	37.31	12.07	9.31	2.71
4 HR	4.92	30.68	9.76	2.68	2.85
8 HR	7.36	21.30	8.58	1.30	4.47
24 HR	14.50	7.65	12.34	1.66	3.71
	% TOTAL DOSE ACCUMULATING IN RAT ORGANS AFTER TREATING TDL WITH 0.01 MG RUTHENIUM RED				
	LN	SPLEEN	LIVER	LUNGS	GUT
2 HR	4.44	36.6	11.05	7.45	3.75
4 HR	6.15	27.8	7.61	3.40	4.08
8 HR	12.07	18.06	5.20	2.23	4.74
24 HR	20.02	6.15	5.00	1.15	5.60

#EACH VALUE REPRESENTS THE MEAN FIGURE DERIVED FROM THE STUDY OF 3-5 RATS AT EACH TIME INTERVAL DESIGNATED.

TABLE 3

THE EFFECTS OF VARYING CONCENTRATIONS OF ALCIAN BLUE DYE UPON IN VITRO LYMPHOCYTE FUNCTIONS

Dose (µg/mL)	Mitogenic# Response	Agglutination	Viability by Trypan Blue	Surface Patches	Caps	Migration* Random	Directional
200	306 cpm	+	10.4%	-	-	-	-
20	182 cpm	±	93.8%	100%	68%	+	+
2	214 cpm	0	96.6%	96%	53%	+	+
0.2	324 cpm	0	95.8%	24%	8%	+	+
Control	268 cpm	0	96.8%	0	0	+	+

#5×10^6 spleen cells cultured for 72 hours with dye and pulsed with ^{3}H-thymidine for 2 hours.

*Random and directional migration scored in modified Nelson assay plates using endotoxin activated serum.

TABLE 4

THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS AFTER INFUSING 300×10^6 ^{3}H-URIDINE LABELLED LYMPHOCYTES TREATED WITH ALCIAN BLUE DYE

Time After Infusion	% of Total Dose Accumulating in Organs after Treating TDL With 200 ug Alcian Blue				
	LN	Spleen	Liver	Lungs	Gut
24 hr	3.72	2.96	16.9	1.30	3.96
	% of Total Dose Accumulating in Organs After Treating TDL With 20 μg Alcian Blue				
24 hr	4.75	1.52	10.65	1.34	6.4
	% of Total Dose Accumulating in Organs After Treating TDL With 2 ug Alcian Blue				
2 hr	4.69	31.74	9.1	6.2	1.91
4 hr	6.31	29.11	6.44	1.92	2.20
8 hr	9.82	20.25	4.21	1.27	2.25
24 hr	18.41	6.72	10.04	1.76	4.92
	% of Total Dose Accumulating in Organs After Treating TDL With 0.2 ug Alcian Blue				
24 hr	18.14	6.65	8.2	1.36	5.28

TABLE 5

THE EFFECTS OF CONCANAVALIN A UPON IN VITRO LYMPHOCYTE FUNCTION

Dose (ug/ml)	Mitogenic# Response	Agglutination	Viability by Trypan Blue	Surface Patches	Caps	Migration* Random	Directional
1	4806 cpm	0	96%	83%	0%	0	0
2.5	1143 cpm	±	86%	94%	3%	0	0
5.0	814 cpm	++	82%	98%	8%	0	0
10.0	263 cpm	++	54%	96%	6%	0	0
Control	218 cpm	0	96.4%	0	0	+	+

#5×10^6 spleen cells cultured for 72 hours with Con A and pulsed with ^{3}H-thymidine for 2 hours.

*Random and directional migration scored by migration under agarose in the modified Nelson chemotactic assay using endotoxin-activated serum.

TABLE 6

THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS AFTER IV INFUSION WITH 300×10^6 ^{3}H-URIDINE LABELED LYMPHOCYTES TREATED WITH CONCANAVALIN A

Time after Injection	% of Total Dose Accumulating in Organs After Treating TDL With 1 ug Con A				
	LN	Spleen	Liver	Lungs	Gut
2 hr	2.93	17.82	14.62	2.30	2.39
4 hr	6.23	30.74	34.65	6.13	5.50
8 hr	9.14	26.62	12.32	2.96	5.42
24 hr	17.70	6.33	7.98	2.36	3.85
	% Total Dose Accumulating in Organs After Treating TDL With 2.5 ug Con A				
	LN	Spleen	Liver	Lungs	Gut
2 hr	1.18	27.40	23.80	3.61	2.21
4 hr	2.09	33.20	18.56	2.30	2.18
8 hr	5.63	31.03	10.02	0.63	1.60
24 hr	9.12	14.90	5.13	0.49	1.39
	% Total Dose Accumulating In Organs After Treating TDL With 5.0 ug Con A				
	LN	Spleen	Liver	Lungs	Gut
2 hr	0.47	1.07	12.73	2.63	2.79
4 hr	0.29	0.62	5.83	1.33	2.30
8 hr	0.27	0.52	5.07	0.43	1.58
24 hr	0.30	0.35	5.26	0.36	1.74
	% Total Dose Accumulating In Organs After Treating TDL With 10 ug Con A				
	LN	Spleen	Liver	Lungs	Gut
2 hr	0.55	4.56	22.40	6.50	1.88
4 hr	0.40	3.94	10.20	3.35	1.70
8 hr	0.69	4.28	7.90	1.41	2.65
24 hr	1.25	2.77	4.23	0.63	1.29

TABLE 7

THE EFFECTS OF ANTI-IMMUNOGLOBULIN UPON IN VITRO LYMPHOCYTE FUNCTION

Dose (mg protein/ml)	Mitogenic# Response	Agglutination	Viability by Trypan Blue	Surface Patches	Caps	Migration* Random	Directional
°Anti IgG 50mg	1648 cpm	0	98.2%	74%	31%	+	+
Anti IgG 5mg	874 cpm	0	97.3%	56%	16%	+	+
Control	262 cpm	0	96.8%	0	0	+	+
=ATG 50mg	106 cpm	0	0	93%	8%	0	0

#5x10^6 spleen cells cultured for 72 hours with anti IgG and pulsed with 3H-thymidine for 2 hours.

*Random and directional migration scored by measuring the migration of Ab-coated lymphocytes under agarose in the modified Nelson assay using endotoxin activated serum as the chemotactic stimulus.

°Rabbit anti rat IgG.

=Rabbit anti rat thymocyte globulin.

TABLE 8

THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS AFTER INFUSING 300×10^6 ^{3}H-URIDINE LABELED LYMPHOCYTES TREATED WITH ANTI-IMMUNOGLOBULIN

TIME AFTER INFUSION	% OF TOTAL INJECTED DOSE ACCUMULATING IN ORGANS				
	LN	SPLEEN	LIVER	LUNG	GUT
2 HR	3.49	29.17	7.38	5.56	2.14
4 HR	5.24	12.16	9.28	5.13	1.59
8 HR	7.46	11.84	6.48	1.90	3.52
24 HR	17.72	7.15	8.95	2.00	2.71

THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS AFTER INFUSING 300×10^6 ^{3}H-URIDINE LABELED LYMPHOCYTES PRE-INCUBATED WITH ANTI-THYMOCYTE GLOBULIN

TIME AFTER INFUSION	% OF TOTAL INJECTED DOSE ACCUMULATING IN ORGANS				
	LN	SPLEEN	LIVER	LUNG	GUT
2 HR	0.33	1.58	10.1	2.1	1.63
4 HR	0.55	1.22	10.9	1.1	2.45
8 HR	0.32	1.42	9.6	0.94	3.82
24 HR	0.31	0.42	11.98	0.62	2.10

TABLE 9

THE EFFECTS OF CYTOCHALASIN A ON LYMPHOCYTES IN VITRO

Dose (µg/ml)	Viability %	Number of Migrating Cells[1]	Distance Migrated[2] Random	Distance Migrated[2] Directional	Ultrastructural Changes: Loss of Microvilli	Ultrastructural Changes: Aggregated Microfilaments	Ultrastructural Changes: Surface Blebs	Ultrastructural Changes: Zeosis
0	98.6	91	40	186	0	0	0	0
0.2	95.8	108	52	201	±	±	0	0
2.0	96.4	80	34	128	+	+	0	±
4.0	96.7	42	20	52	++	++	+	+
6.0	97.2	0	0	0	++++	++++	++	++
8.0	94.8	0	0	0	++++	++++	++	++

[1] Mean number of migrating cells in a 66µ wide strip connecting the centers of all 3 wells in agarose plates.

[2] Mean linear distance in microns traveled in 12 hours by lymphocyte populations showing random movement and directional migration towards endotoxin activated serum in agarose plates.

TABLE 10

THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS AFTER IV INFUSION WITH 300×10^6 ^{3}H-URIDINE LABELED LYMPHOCYTES TREATED WITH CYTOCHALASIN A

Time After Injection	LN	Spleen	Liver	Lungs	Gut
	% of Total Dose In Organs After Treating Cells With 10 μg/ml				
2 hr	0.07	23.2	13.3	4.0	2.1
4 hr	0.14	13.6	9.3	1.0	3.9
8 hr	0.14	4.0	7.0	0.6	2.6
24 hr	0.15	2.0	7.5	0.1	3.9
	% of Total Dose In Organs After Treating Cells With 6 μg/ml				
	LN	Spleen	Liver	Lungs	Gut
2 hr	0.18	11.4	16.2	7.3	2.2
4 hr	0.19	8.3	6.6	6.8	7.2
8 hr	0.19	3.9	7.3	1.7	2.3
24 hr	0.36	2.8	7.3	4.8	3.8
	% of Total Dose In Organs After Treating Cells With 4 μg/ml				
	LN	Spleen	Liver	Lungs	Gut
2 hr	0.32	9.7	9.8	4.1	3.3
4 hr	0.22	6.9	7.3	8.9	5.3
8 hr	0.29	3.9	7.8	1.8	2.9
24 hr	0.48	2.0	5.3	2.7	3.9
	% of Total Dose In Organs After Treating Cells With 2 μg/ml				
	LN	Spleen	Liver	Lungs	Gut
2 hr	2.73	14.3	11.2	13.5	4.1
4 hr	2.50	12.9	8.9	3.8	2.3
8 hr	5.05	10.7	12.5	4.3	2.5
24 hr	9.08	4.7	5.2	2.3	3.8

#Each point = mean value derived from studying 3-5 rats.

TABLE 11

THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS AFTER IV INFUSION

WITH 300×10^6 ^3H-URIDINE LABELED LYMPHOCYTES TREATED WITH CYTOCHALASIN B AT 10 μG/ML

Time After Injection	% of Total Dose In Organs				
	LN	Spleen	Liver	Lungs	Gut
½ hr	1.57	22.1	15.7	13.8	2.1
2 hr	1.84	18.8	6.3	3.2	2.3
4 hr	1.56	12.9	10.0	3.6	0.9
8 hr	2.92	9.5	7.8	1.7	1.8
24 hr	5.79	3.5	13.3	3.7	1.5

#Each point = mean value derived from the study of 3-5 rats.

TABLE 12

THE IN VITRO EFFECTS OF COLCHICINE UPON RAT THORACIC DUCT LYMPHOCYTES

Drug Dose	Viability by Trypan Blue Exclusion	Random Motility	Traffic Kinetics (% of Total Dose of Injected Radioactivity in Lymph Nodes 24 hours After Infusing 3×10^8 Cells Labeled With 3H-Uridine)#	
			Mean	Range
10^{-2} colc	64.3%	0	6.12	(2.1-8.3)
10^{-3} colc	78.8%	0	6.48	(5.3-7.4)
10^{-4} colc	94.6%	+	16.56	(12.8-19.4)
10^{-6} colc	98.2%	+	18.21	(17.1-21.2)
10^{-8} colc	95.3%	+	17.85	(16.9-20.8)
10^{-10} colc	94.4%	+	17.54	(17.8-22.3)
10^{-4} LumiColc	96.8%	+	22.41	(17.3-24.1)
No drug	95.4%	+	19.80	(16.8-23.4)

#Each point = mean value observed in study of 3-5 rats

TABLE 13

THE ACCUMULATION OF RADIOACTIVITY (% OF TOTAL DOSE INJECTED) IN ORGANS OF NORMAL RATS INFUSED WITH 300×10^6 ^{3}H-URIDINE LABELED LYMPHOCYTES TREATED WITH COLCHICINE FOR ½ HOUR#

Time After Infusion	LN	Spleen	Liver	Lungs	Gut
Cells Pre-Incubated With 10^{-4}M Colchicine					
2 hr	1.90	38.2	9.6	9.6	3.90
4 hr	1.95	17.3	5.9	2.4	1.43
8 hr	3.39	15.6	4.6	1.3	1.65
18 hr	9.72	5.2	5.8	0.8	1.60
Cells Pre-Incubated With 10^{-5}M Colchicine					
2 hr	1.63	22.1	12.9	15.7	1.23
4 hr	1.74	15.7	5.4	4.4.	1.03
8 hr	2.58	18.6	5.5	1.7	1.42
18 hr	10.90	5.7	4.7	0.6	1.26
Cells Pre-Incubated With 10^{-6}M Colchicine					
2 hr	2.55	24.1	6.9	3.9	2.17
4 hr	3.27	22.5	5.5	1.6	2.50
8 hr	5.91	16.4	3.3	1.0	2.90
18 hr	10.90	4.3	3.9	0.6	2.2
Cells Pre-Incubated With 10^{-8}M Colchicine					
2 hr	4.10	29.7	4.4	4.1	2.25
4 hr	6.56	25.5	4.7	2.1	3.05
8 hr	8.72	25.3	2.1	3.6	2.84
18 hr	13.19	7.2	4.6	0.8	2.70
Untreated Cells					
2 hr	3.86	43.0	9.25	5.64	2.34
4 hr	5.67	30.1	7.51	1.80	2.83
8 hr	7.63	12.7	5.76	1.31	2.11
18 hr	12.54	5.7	6.10	0.67	1.25

#Each value represents the mean derived from study of 3-5 rats.

TABLE 14

THE ACCUMULATION OF RADIOACTIVITY (% TOTAL DOSE INJECTED) IN THE ORGANS OF NORMAL RATS INFUSED WITH 300x10^{6} 3H-URIDINE LABELED LYMPHOCYTES PRE-TREATED WITH LUMICOLCHICINE#

Time After Infusion	LN	Spleen	Liver	Lungs	Gut
	% Uptake Using Cells Treated With 10^{-4}M Lumicolchicine				
½ hr	3.61	15.04	30.40	42.17	2.17
2 hr	4.36	26.51	11.28	5.82	1.83
4 hr	4.89	22.87	8.17	2.42	1.65
8 hr	7.68	19.42	7.47	1.58	2.81
24 hr	20.11	7.26	6.53	1.43	3.58
	% Uptake Using Cells Treated With 10^{-5}M Lumicolchicine				
	LN	Spleen	Liver	Lungs	Gut
½ hr	3.78	20.29	28.43	37.62	2.80
2 hr	5.46	46.92	13.19	6.58	4.51
4 hr	7.28	35.60	8.33	3.93	2.85
8 hr	12.20	28.42	8.79	2.17	5.88
24 hr	24.29	5.77	10.72	3.70	4.40
	% Uptake Using Untreated Cells				
	LN	Spleen	Liver	Lungs	Gut
½ hr	3.80	25.86	26.98	35.84	2.97
2 hr	4.62	25.27	14.64	5.52	2.34
4 hr	4.66	27.35	9.07	2.59	1.98
8 hr	6.35	14.85	4.93	1.39	1.93
24 hr	20.76	6.68	6.84	1.27	3.71

#Each point = mean value derived from the study of 3-5 rats.

TABLE 15

COMPARISON OF THE INTRA-NODAL DISTRIBUTION OF NORMAL AND COLCHICINE-TREATED RADIOLABELED LYMPHOCYTES BY AUTORADIOGRAPHY AT SEQUENTIAL INTERVALS AFTER TRANSFUSION INTO NORMAL RECIPIENTS

Time After Injection	HEV Lumen NL	HEV Lumen COLC	HEV Wall and Sheath NL	HEV Wall and Sheath COLC	10 HPF of Diffuse Cortex NL	10 HPF of Diffuse Cortex COLC	Medullary Sinuses NL	Medullary Sinuses COLC	Total NL	Total COLC
3 MIN	10.1	1.8	15.8	0.3	0.2	0	1.0	0	27	2.1
30 MIN	1.8	3.6	27.8	4.1	28.8	1.4	1.2	0	59.6	9.1
1 HR	0.4	4.2	12.1	6.4	150.8	11.7	10.2	1.3	173.4	23.5
4 HR	2.2	0.8	12.0	0.9	302.8	10.0	25.6	1.4	342.6	13.1
24 HR	4.0	1.5	22.3	1.0	406.2	304.1	23.3	26.3	455.4	332.8

= OBSERVATIONS ON SUBMANDIBULAR NODES EACH VALUE IS THE MEAN DERIVED FROM STUDY OF 3-5 DIFFERENT NODES.

TABLE 16

THE ACCUMULATION OF RADIOACTIVITY (% OF TOTAL DOSE INJECTED) IN PERIPHERAL LYMPH NODES AFTER INFUSING ^{3}H-URIDINE LABELED THORACIC DUCT LYMPHOCYTES DERIVED FROM COLCHICINE TREATED RATS INTO NORMAL RECIPIENTS

Hours After Cell Infusion	Nodal Uptake of Radioactivity # — TDL From Colchicine Rats	TDL From Normal Rats
½	0.40	1.96
2	0.43	3.80
4	0.50	5.60
8	0.78	8.31
18	3.45	12.04

= Each value represents mean derived from study of 3-5 rats.

TABLE 17

THE ACCUMULATION OF RADIOACTIVITY IN ORGANS OF RATS INFUSED WITH ^{3}H-URIDINE LABELED LYMPHOCYTES ONE HOUR AFTER IP INJECTION WITH COLCHICINE#

Time After Injection	LN	Spleen	Liver	Lungs	Gut
		Colchicine Dose of 1 mg/Kg			
2 hr	0.61	5.5	18.3	15.7	5.1
4 hr	0.94	9.3	17.0	8.6	6.1
8 hr	2.23	10.3	14.3	3.4	7.7
24 hr	2.18	2.9	6.3	0.9	4.9
		Colchicine Dose of 0.5 mg/Kg			
	LN	Spleen	Liver	Lungs	Gut
2 hr	1.35	13.3	14.1	9.1	1.95
4 hr	1.82	8.1	13.8	4.0	2.61
8 hr	4.10	8.1	13.6	2.9	5.43
24 hr	6.24	3.0	8.4	1.1	2.91
		Colchicine Dose of 0.1 mg/Kg			
	LN	Spleen	Liver	Lungs	Gut
2 hr	2.01	19.1	7.5	13.3	1.1
4 hr	2.45	7.6	3.9	1.6	12.1
8 hr	3.31	7.6	5.8	2.6	10.4
24 hr	7.85	4.3	6.6	1.4	2.6
		Saline Only IP			
	LN	Spleen	Liver	Lungs	Gut
2 hr	2.4	28.3	14.9	5.6	3.0
4 hr	4.1	24.5	6.2	1.2	3.2
8 hr	7.3	16.8	6.3	1.4	9.1
24 hr	14.6	6.2	5.1	0.8	6.5

#In each study colchicine was injected one hour before and 8 hours after cell transfusion to maintain uninterrupted pharmacologic effects due to drug turnover *in vivo*.

TABLE 18

THE ACCUMULATION OF RADIOACTIVITY (% TOTAL DOSE INJECTED) IN RAT ORGANS

24 HOURS AFTER INFUSING 300×10^6 ^{3}H-URIDINE LABELED LYMPHOCYTES TREATED WITH VINCRISTINE

	% Uptake				
Dose of Vincristine	LN	Spleen	Liver	Lungs	Gut
None	14.97	16.01	11.03	1.72	6.21
10^{-10}M	12.01	19.44	10.00	1.54	5.33
10^{-8}M	11.67	17.83	12.20	1.69	4.77
10^{-6}M	9.88	15.28	10.31	1.60	3.97
10^{-4}M	9.76	16.22	11.33	1.58	4.78

#Each value = mean derived from study of 3-5 rats.

TABLE 19

CHEMOTACTIC RESPONSES OF DIFFERENT RAT LEUKOCYTE POPULATIONS TO ENDOTOXIN-ACTIVATED SERUM

Type of Lymphocyte	Population Distribution# by Stem-Leaf Plotting	Chemotactic Index (A/B)	Chemotactic Differential (A-B)
Normal TDL	Shift towards A	4.3	132 microns
T-Blasts	shift towards A	5.4	1130 microns
B-Blasts	Random	1.4	21 microns
Lymphoma 8	Random	1.2	33 microns
Granulocytes	Shift towards A	6.9	1280 microns
Macrophages	Shift towards A	4.0	1060 microns

#A = cell movement from center well towards outer well containing chemotactic factor

B = cell migration from center well toward inner well containing control solution

TABLE 20

CHEMOTACTIC RESPONSES OF NORMAL RAT THORACIC DUCT LYMPHOCYTES (TDL) INCUBATED WITH CON A AND THEN EXPOSED TO A GRADIENT OF ENDOTOXIN-ACTIVATED SERUM

Type of Cells	Population Distribution by Stem-Leaf Plotting	Chemotactic Index (A/B)	Chemotactic Differential (A-B)
Normal TDL	shift towards A	4.3	132 microns
Con A-TDL at 24 hours	no movement	0	0
Con A-TDL at 36 hours	no movement	0	0
Con A-TDL at 48 hours	shift towards A	6.8	1390 microns

TABLE 21

THE IDENTITY OF THE LYMPHOCYTE CHEMOATTRACTANT IN ENDOTOXIN-ACTIVATED SERUM

TYPE OF CELL	TYPE OF SERUM#	POPULATION DISTRIBUTION BY STEM-LEAF PLOTTING	CHEMOTACTIC INDEX (A/B)	CHEMOTACTIC DIFFERENTIAL (A-B)
GRANULOCYTES	NS	RANDOM	1.1	23 MICRONS
GRANULOCYTES	ENS	SHIFT TOWARDS A	6.9	1280 MICRONS
GRANULOCYTES	HNS+E	RANDOM	1.3	34 MICRONS
GRANULOCYTES	ENS+H	SHIFT TOWARDS A	6.0	1210 MICRONS
GRANULOCYTES	CVS+E	RANDOM	0.9	-30 MICRONS
MACROPHAGES	NS	RANDOM	1.0	0 MICRONS
MACROPHAGES	ENS	SHIFT TOWARDS A	4.0	1060 MICRONS
MACROPHAGES	HNS+E	RANDOM	1.3	30 MICRONS
MACROPHAGES	ENS+H	SHIFT TOWARDS A	4.1	1018 MICRONS
MACROPHAGES	CVS+E	RANDOM	1.1	18 MICRONS
TDL	NS	RANDOM	0.9	-16 MICRONS
TDL	ENS	SHIFT TOWARDS A	4.3	132 MICRONS
TDL	HNS+E	RANDOM	1.1	24 MICRONS
TDL	ENS+H	SHIFT TOWARDS A	3.9	120 MICRONS
TDL	CVS+E	SHIFT TOWARDS A	3.2	98 MICRONS
T-BLASTS	NS	RANDOM	1.2	60 MICRONS
T-BLASTS	ENS	SHIFT TOWARDS A	5.4	1130 MICRONS
T-BLASTS	HNS+E	RANDOM	0.9	-46 MICRONS
T-BLASTS	ENS+H	SHIFT TOWARDS A	5.0	1080 MICRONS
T-BLASTS	CVS+E	SHIFT TOWARDS A	5.5	1166 MICRONS

#NS = NORMAL RAT SERUM
ENS = ENDOTOXIN ACTIVATED NORMAL SERUM
HNS+E = NORMAL SERUM HEATED AT 56°C FOR 20 MINUTES THEN TREATED WITH ENDOTOXIN
ENS+H = ENDOTOXIN ACTIVATED SERUM HEATED AT 56°C FOR 20 MINUTES BEFORE USE
CVS+E = SERUM FROM RATS DEPLETED OF $C'_3 - C'_9$ BY TREATMENT WITH COBRA VENOM FACTOR AND ACTIVATED BY ENDOTOXIN

TABLE 22

THE ACCUMULATION OF RADIOACTIVITY (% DOSE INJECTED) IN THE ORGANS OF VENOM-TREATED RATS AT SEQUENTIAL INTERVALS AFTER INFUSING 300×10^6 ^3H-URIDINE LABELED LYMPHOCYTES

	% Uptake in Venom-Treated Rats				
Time After Injection	LN	Spleen	Liver	Lungs	Gut
2 hr	3.59	25.19	9.99	7.83	4.79
4 hr	4.09	18.37	8.25	5.10	4.13
8 hr	8.62	17.92	5.16	2.36	6.50
24 hr	9.89	5.74	4.22	0.94	5.28

	% Uptake in Saline-Treated Controls				
	LN	Spleen	Liver	Lungs	Gut
2 hr	3.47	18.81	7.12	9.21	2.55
4 hr	5.95	20.83	6.62	2.93	4.81
8 hr	6.65	16.33	5.29	2.31	4.40
24 hr	10.12	6.01	4.75	0.95	5.41

#Each value = mean derived from study of 2-3 rats.

TABLE 23

COMPARISON OF THE CHEMOTACTIC RESPONSES+ OF RAT LEUKOCYTES TO OTHER KNOWN CHEMOTACTIC FACTORS

TYPE OF CELL	*E.coli* CULTURE FILTRATE	SUPERNATANTS FROM A23187-TREATED BUFFY COAT CELLS	CHEMOTACTIC* TRIPEPTIDE OF SCHIFFMAN
GRANULOCYTES	+	+	+
MACROPHAGES	0	+	±
TDL	0	0	0
T-BLASTS	0	0	0
B-BLASTS	0	0	0
LYMPHOMA 8	0	0	0

+ = CHEMOTACTIC INDEX OF 2 OR MORE AND/OR CHEMOTACTIC DIFFERENTIAL OF 100 OR MORE MICRONS.

* = F MET-LEU-PHE AT CONCENTRATIONS RANGING FROM 10^{-6} - 10^{-10}M.

TABLE 24

THE ABILITY OF LYMPHOCYTES TO SECRETE FACTORS CAUSING DIRECTIONAL MIGRATION OF OTHER LYMPHOID SUBPOPULATIONS

Migrating Cell type	Cell Source of Chemotactic Stimulus	Directional Movement	Chemotactic Index	Chemotactic Differential
B-blasts	B-blasts	random	1.1	30 u
T-blasts	B-blasts	towards B cells	3.8	960 u
TDL	B-blasts	random	0.9	-16 u
Ly 8	B-blasts	random	1.2	64 u
B-blasts	T-blasts	random	0.8	-48 u
T-blasts	T-blasts	random	1.0	10 u
TDL	T-blasts	random	1.1	16 u
Ly 8	T-blasts	random	1.3	72 u
B-blasts	TDL	random	1.1	18 u
T-blasts	TDL	random	0.8	-56 u
TDL	TDL	random	1.0	12 u
Ly 8	TDL	random	0.7	-88 u

TABLE 25

COMPARISON OF REGIONAL AND CONTRALATERAL AXILLARY LYMPH NODE WEIGHTS IN RATS INJECTED SQ WITH COMPLETE FREUND'S ADJUVANT

Days After CFA Injection	Regional		Contralateral	
	Mean	Range	Mean	Range
0	18	(16-21)	17	(14-21)
1	38	(24-65)	21	(13-28)
3	41	(30-56)	21	(14-25)
7	53	(28-90)	25	(15-33)
14	47	(26-63)	23	(11-30)
28	38	(26-58)	23	(13-28)
56	34	(29-42)	24	(18-27)

= Each point represents the mean of 10 rats studied.

TABLE 26

PERIPHERAL LYMPHATIC TISSUE WEIGHT AT SEQUENTIAL INTERVALS AFTER CHALLENGE WITH VEE IN COMPLETE CFA

	CFA Injected Rats #		Saline Injected Controls #	
Days After Injection	Lymph Node Wt	Spleen Wt	Lymph Node Wt	Spleen Wt
0	760	482	784	494
1	857	456	---	---
3	762	457	---	---
7	892	524	801	513
14	884	551	---	---
28	959	443	749	484
56	1187	519	819	533

= Each value represents mean derived from the study of 10 rats.

TABLE 27

SEQUENTIAL CHANGES IN T AND B-CELL ZONES OF REGIONAL LYMPH NODES AFTER IMMUNIZATION WITH VEE IN COMPLETE FREUND'S ADJUVANT#

Days After Challenge	Lymph Node Weight (mg)	Cortical Area by Planimetry	% of Total Node Composed by Cortex	% of Total Node Composed by GC	Cortical Weight (mg)	GC Weight (mg)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean	Mean
0	18 ± 3.3	2.3 ± 0.9	56.5 ± 4.0	2.0 ± 1.1	10.17	0.36
1	38 ± 4.2	2.7 ± 1.1	56 ± 7.5	2.2 ± 1.3	21.28	0.88
3	41 ± 5.4	4.5 ± 1.1	64 ± 8.0	2.9 ± 1.8	32.24	1.19
7	53 ± 6.7	5.6 ± 0.6	70 ± 4.4	2.3 ± 2.6	37.10	1.22
14	47 ± 4.8	3.8 ± 1.9	65 ± 9.6	3.4 ± 3.5	30.55	1.60
28	38 ± 5.1	3.5 ± 1.7	70 ± 7.5	5.7 ± 4.3	26.60	2.15
56	34 ± 4.2	---	---	---	---	---

= Each value represents the mean derived from study of 6-8 rats.

TABLE 28

SUMMARY OF THE RADIOKINETIC AND MORPHOLOGIC CHANGES APPEARING IN REGIONAL NODES DRAINING INJECTION SITES OF VEE VACCINE IN CFA

Day After Challenge	Mean Node Weight (mg)	LMI# (mean)	TI* (mean)	Lymphocyte "Plugs"	Granuloma Formation	Cortical Mass (mg)	Medullary Mass (mg)
0	18	0.72	0.94	0	0	10.2	7.8
1	38	1.20	1.86	±	0	21.3	15.8
3	41	1.24	3.03	++	0	32.2	7.6
7	53	1.32	2.24	++	0	37.1	14.7
14	47	1.14	3.23	+	+	30.6	14.8
28	38	1.08	1.63	+	+	26.6	9.3
56	34	--	1.17	±	+	--	--

= LMI = $\frac{\text{NUMBER OF MIGRATING LYMPHOCYTES}}{\text{NUMBER OF ENDOTHELIAL CELLS}}$ COUNTED IN HISTOLOGIC SECTIONS.

* = TI = $\frac{\text{TOTAL DPM IN REGIONAL NODE}}{\text{TOTAL DPM IN CONTRALATERAL NODE}}$ AT 24 HOURS AFTER INFUSING 3×10^8 ^{3}H-URIDINE LABELED TDL CELLS IV.

TABLE 29

COMPARISON OF THE ACCUMULATION OF RADIOACTIVITY IN AXILLARY LYMPH NODES AFTER INJECTING ^{3}H-URIDINE LABELED LYMPHOCYTES INTO CFA TREATED RATS

Days After CFA Injection	Regional Node Uptake (% of total dose)	Trapping Index (CPM RN/CPM in CN)	Contralateral Node Uptake (% total dose)
0	.31	.94	.33
1	.65	1.86	.35
3	1.15	3.03	.38
7	.85	2.24	.35
14	1.15	3.23	.40
28	.52	1.63	.32
56	.34	1.17	.29

TABLE 30

COMPARISON OF THE ACCUMULATION OF RADIOACTIVITY IN THE TOTAL MASS OF ORGANIZED LYMPHATIC TISSUE OF CFA-TREATED RATS #

Days After CFA Injection	Lymph Node Uptake + (% of dose injected)		Spleen Uptake (% of dose injected)	
	Mean	(Range)	Mean	(Range)
0	13.1	(12-14.5)	5.3	(3-8)
1	12.5	(12-13)	6.65	(5-8)
3	11.5	(10-12.8)	5.81	(5-6.6)
7	9.1	(8-10.5)	7.1	(6-7.5)
14	14.1	(13-14.7)	5.4	(5-6)
28	12.2	(11-13)	4.3	(4.1-5.0)
56	9.3	(8-11)	2.5	(1-3)

= Each value represents the mean derived from the study of 3-5 rats.

+ = All values represent the total uptake at 24 hours post-transfusion with labeled lymphocytes.

TABLE 31

THE EFFECTS OF ADJUVANTS UPON ANTIBODY RESPONSES TO VEE VACCINE

Type of Rat	Immunization Procedure		Plaque Neutralization Titers 2 wks	4 wks	6 wks	8 wks	12 wks	20 wks
Normal	VEE	$\bar{x}$	106	557	403	640	320	80
		SD	1.0	2.1	1.0	1.1	1.5	1.1
		SE	0.0032	0.0067	0.033	0.033	0.048	0.032
Thymex	VEE	$\bar{x}$	1470	1463	422	840	277	212
		SD	1.0	1.7	3.1	6.0	4.1	3.2
		SE	0.0032	0.0054	0.0097	0.019	0.013	0.015
Normal	VEE	$\bar{x}$	3378	6629	5090	3599	3578	560
	+	SD	1.0	1.2	1.3	1.5	1.4	2.1
	CFA	SE	0.0032	0.0037	0.0131	0.0145	0.0135	0.0150
Thymex	VEE	$\bar{x}$	13,195	15,229	8830	7687	5072	1760
	+	SD	1.0	1.1	1.1	1.1	1.2	1.4
	CFA	SE	0.0032	0.0034	0.0035	0.0034	0.0036	0.0145
Normal	VEE	$\bar{x}$	844	422	320	320	184	116
	into	SD	1.1	3.8	2.0	2.7	2.8	1.6
	thymus	SE	0.0036	0.0120	0.0062	0.0085	0.0086	0.0072
Normal	VEE	$\bar{x}$	320	159	35	26	17	< 10
	+	SD	2.0	3.5	2.5	1.6	1.4	--
	Digitonin	SE	0.0062	0.0109	0.0078	0.0049	0.0044	--
Thymex	VEE	$\bar{x}$	80	106	80	139	61	83
	+	SD	1.6	2.0	2.4	2.6	2.9	2.3
	Digitonin	SE	0.0162	0.0064	0.0076	0.0082	0.0092	0.0066

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This report demonstrates that a variety of different ligands such as polycationic dyes, concanavalin A and anti-immunoglobulin aggregate outer membrane components to form patches and caps visible by transmission electron microscopy. At high ligand concentrations, this results in "blindfolding" effects which disrupt lymphocyte recirculation by preventing the treated cells from attaching to HEV surfaces. _In vitro_ observations indicate that these membrane-ligand complexes are slowly cleared from lymphocyte surfaces by
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20. ABSTRACT (CON'T.)
SHEDDING AND ENDOCYTOSIS. HOWEVER, WHEN SUCH CELLS ARE TRANSFUSED INTO NORMAL RECIPIENTS, THE SURFACE LIGANDS ARE STRIPPED FROM THE LYMPHOCYTE MEMBRANE AS THESE CELLS EMIGRATE ACROSS HEV WALLS BY SELECTIVE, SEGMENTAL PHAGOCYTIC ACTIVITY OF THE ADJACENT ENDOTHELIAL CELLS. THESE OBSERVATIONS SUGGEST THAT A MAJOR PHYSIOLOGIC FUNCTION OF HIGH-ENDOTHELIAL CELLS IS TO "SCRUB" THE SURFACES OF EMIGRATING LYMPHOCYTES AND REMOVE ADSORBED PROTEINS OR IRRELEVANT ANTIGENS BEFORE THE LYMPHOCYTES ENTER THE NODAL PARENCHYMA WHERE THE CRITICAL SEQUENCE OF ANTIGEN RECOGNITION AND CELLULAR COLLABORATION OCCUR. RELATED STUDIES WITH COLCHICINE AND THE CYTOCHALASINS PROVIDE THE FIRST EVIDENCE INDICATING THAT THE SURFACE RECOGNITION MECHANISMS REQUIRED FOR LYMPHOCYTE "HOMING" INTO HEV ARE LINKED TO THE CYTOSKELETON THROUGH TRANSMEMBRANE RESTRAINTS. POSTULATES THAT THE ENTRY AND SUBSEQUENT REDISTRIBUTION OF LYMPHOCYTE SUBPOPULATIONS INTO DIFFERENT ZONES WITHIN LYMPH NODES MAY BE CONTROLLED BY CHEMOTAXIS ARE SUPPORTED BY DEMONSTRATIONS THAT: LYMPHOCYTES EXHIBIT TRUE CHEMOTACTIC RESPONSES IN VITRO; SELECTIVE LYMPHOCYTE CHEMOATTRACTANTS ARE GENERATED BY ENDOTOXIN ACTIVATION OF SERUM DEPLETED OF $C'_3 - C'_9$ BY TREATMENT WITH COBRA VENOM FACTOR, AND THAT LYMPHOCYTE SUBCLASSES DISPLAY DIRECTIONAL MIGRATION TO DIFFERENT CHEMOTACTIC AGENTS (I.E.: B-BLASTS ELABORATE FACTORS WHICH ATTRACT T-BLASTS).

STUDIES ON THE POTENTIATION OF IMMUNE RESPONSES TO VEE VACCINE BY COMPLETE FREUND'S ADJUVANT INDICATE THAT AGENT CAUSES PROLONGED LYMPHOCYTE TRAPPING AND GRANULOMA FORMATION IN THE REGIONAL NODE CONSISTENT WITH RE STIMULATION WHICH ARE FOLLOWED BY NODAL ENLARGEMENT BY PROMINENT GERMINAL CENTERS AND EXPANDED MEDULLARY CORDS. OF EQUAL INTEREST ARE DEMONSTRATIONS THAT THYMECTOMY PRIOR TO CHALLENGE WITH VEE VACCINE ALONE OR IN COMBINATION WITH ADJUVANTS RESULTS IN GREATER AND MORE PROLONGED ANTIBODY RESPONSES THAN ARE SEEN BY COMPARABLE IMMUNIZATION OF INTACT ANIMALS. THESE RESULTS SUGGEST THAT THE ABROGATION OF T-CELL SUPPRESSOR EFFECTS MAY PROVIDE ANOTHER MEANS FOR AUGMENTING IMMUNE RESPONSES INDUCED BY VACCINES AND ADJUVANTS.